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Use of Small Molecules to Study Invasion and Egress in Toxoplasma gondii.

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USE OF SMALL MOLECULES TO STUDY INVASION AND EGRESS IN *TOXOPLASMA GONDII*.

A Thesis Presented

by

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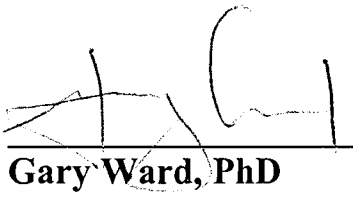

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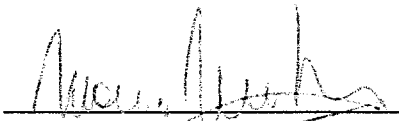

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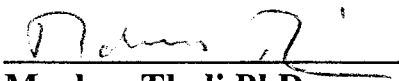

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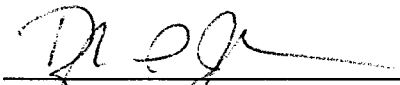

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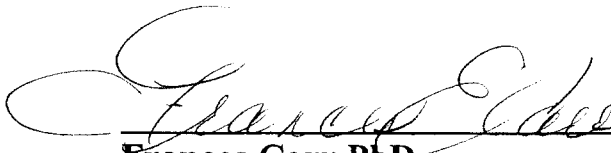

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Abstract

Toxoplasma gondii is an obligate intracellular pathogen, and belongs to the phylum Apicomplexa. The pathogenesis of the parasite is thought to be due to, in part, repeated cycles of invasion of host cells, replication and lysis of host cells. *T.gondii* is a haploid organism and forward genetic methods to study invasion are hard to employ, because if genes essential to invasion are disrupted, they will be lethal to the parasite. Although conditional mutants have been generated, it is hard to generate a large library of conditional mutants. As an alternate approach, a library of small molecules can be screened in an assay for a particular phenotype. Then the target of the small molecule responsible for the phenotype can be identified. Two different small molecules screens can be performed: a non-presumptive approach using small molecules with no known targets or a hypothesis based approach using small molecules with known or hypothesized targets. Approximately 12,000 structurally diverse small molecules with no known target were tested in a high throughput screen in our lab to identify inhibitors of *T.gondii* invasion.

In this thesis I have described two different projects using small molecule inhibitors identified from this screen. My first project was hypothesis driven. One of the inhibitors identified, 118793, is structurally similar to a known inhibitor of mammalian cGMP Phosphodiesterase. The goal of the project was to test the hypothesis that cGMP Phosphodiesterase is the target of the compound 118793 during invasion. This hypothesis is also supported by the fact, that cGMP dependent protein kinase is important for invasion and motility of *T.gondii*. An in-vitro phosphodiesterase assay using tritiated cGMP as substrate and anion exchange chromatography was adopted to test the hypothesis. The effect of compound 118793 on the cGMP hydrolytic activity of phosphodiesterase in *T.gondii* extracts and purified bovine phosphodiesterase was tested. From our assay, compound 118793 does not appear to inhibit cGMP phosphodiesterase in *T.gondii* or purified cGMP Phosphodiesterase. In addition, I also generated a FRET based cGMP indicator called CYGNET 2.1 to test the hypothesis *in vivo*.

The second project was to test the effect of the invasion inhibitors identified in our screen on *T.gondii* egress. The dogma in the field is that the process of invasion and egress are mechanistically similar. I thought it would of interest to determine if any of the small molecules inhibited invasion but not egress. I adopted an ionophore induced egress assay to address this question. Ionophore-induced egress is thought to involve active motility. I tested invasion inhibitors that inhibit motility and some that do not inhibit motility. From my assay, it appears that one of the motility inhibitors does not inhibit egress. In addition, two of the non-motility inhibitors inhibit egress. These results prompt us to revisit current theories about invasion and egress.

Given the limitations we have to study *T.gondii*, small molecule approach has proven to be very useful and advantageous in identifying proteins and pathways involved in invasion.

Acknowledgements

I want to sincerely thank my advisor and mentor, Dr. Gary Ward, for his unconditional support, his patience and his confidence in my capacity to succeed. The three years I spent in the lab were an incredible learning experience. It has been a pleasure and an honor to work under his supervision.

I want to thank the members of my studies committee- Drs. Mariana Matrajt, Markus Thali, Douglas Johnson and Wolfgang Dostmann- for their continuous support and suggestions over the past years.

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CHAPTER I

Introduction

Toxoplasma gondii is an obligate intracellular parasite that can infect almost all warm-blooded vertebrates including man. *T. gondii* is the causative agent of toxoplasmosis. In most cases, toxoplasmosis is asymptomatic because of effective protective immunity in healthy individuals. However, it may prove fatal in immunocompromised people. Toxoplasmosis, when transmitted congenitally from mother to the unborn fetus, may result in severe birth defects and death in some cases. *T. gondii* belongs to the phylum Apicomplexa and is an excellent model for studying other apicomplexans such as *Plasmodium* (causes malaria) and *Cryptosporidium* (causes cryptosporidosis) (Roos, Crawford et al. 1999; Kim and Weiss 2004).

The life cycle of *T. gondii* includes both a sexual and an asexual phase. The sexual phase takes place in the definitive hosts, which are all members of the family Felidae (*e.g.*, domestic cats). During the sexual phase, oocysts develop in the intestine of the cat. The oocysts are shed in the cat faeces, and contain the highly invasive sporozoites. Animals and humans are the intermediary hosts and are infected by ingesting food and water contaminated by oocysts shed in cat feces. The asexual phase of the life cycle takes place in any warm-blooded intermediate host ingesting the oocyst. Once inside the intermediate host, the tachyzoites, which are the fast growing forms, begin the asexual life cycle. They cause acute infections, however the host immune response clears

the infection effectively. Cytokines such as IL-12 and IFN-γ are produced by the dendritic, natural killer and T cells of the host (Yap and Sher 1999). In immunocompromised people such as people with AIDS, the parasites continue replicating as tachyzoites within a vacuole known as the parasitophorous vacuole (PV), surrounded by the parasitophorous vacuole membrane (PVM).

However, in immunocompetent hosts, the host immune response to the primary infection forces the tachyzoites to switch into bradyzoites. The bradyzoites are the slow growing forms and replicate within the host cells to form tissue cysts. Intermediate hosts can also be infected by ingesting these tissue cysts in unwashed vegetables, or improperly cooked meat. The life cycle of *T. gondii* completes a full circle when cats ingest these tissue cysts. Tissue cysts can grow in any organ, but are predominant in the brain and muscles. As they are the resistant to the host immune response, bradyzoites cause a chronic infection. In immunocompromised patients with a weak immune system the bradyzoites switch back into tachyzoites and the cycle continues. Small populations of bradyzoites spontaneously switch back to tachyzoites in immunocompetent individuals as well.

The apical end of the *T. gondii* consists of the conoid, two intraconoid microtubules, and two polar rings. The conoid is made of a unique polymer form of tubulin (Hu, Roos et al. 2002). It looks like a compressed spring and it repeatedly protrudes and retracts during motility. Conoid protrusion can be induced using calcium ionophores (Mondragon and Frixione 1996; Monteiro, de Melo et al. 2001). The actual function of the conoid is yet unknown.

In the apical end of the parasite, there are two types of secretory organelles known as the micronemes and the rhoptries. The micronemes are tubular structures and the microneme proteins contain conserved domains, especially thrombospondin-like motifs, which suggest that they may play an important role in adhesion (Wan, Carruthers et al. 1997). TgMIC 1-4 have been shown to bind to host cells (Fourmaux, Achbarou et al. 1996). Microneme proteins are secreted by extracellular parasites constitutively (Wan, Carruthers et al. 1997) and secretion can be upregulated using calcium ionophores (Carruthers and Sibley 1999). The above observation and other studies (Carruthers, Moreno et al. 1999; Carruthers and Sibley 1999; Lovett, Marchesini et al. 2002; Lovett and Sibley 2003) have shown that intracellular calcium levels of parasites affect the secretion of microneme proteins. During invasion, it has been shown that secretion of microneme proteins increases (Carruthers and Sibley 1997) and that they have to undergo proteolytic processing for proper targeting and function (Brossier, Jewett et al. 2005; Teo, Zhou et al. 2007). TgMIC2 is a very well studied microneme protein. It is a transmembrane protein and is secreted to the surface of the parasite during invasion. It is translocated to the posterior of the parasite as it enters the host cell. At or near the posterior end of the parasite TgMIC2 is cleaved within its transmembrane domain by rhomboid proteases (Dowse and Soldati 2004; Dowse, Pascall et al. 2005). This intramembrane cleavage results in the shedding of the extracellular domain of TgMIC2.

TgMIC 3,5 and 6 are proteolytically cleaved intracellularly during transport to the micronemes (Brydges, Sherman et al. 2000; Opitz, Di Cristina et al. 2002; Brydges, Zhou

et al. 2006). TgMIC4 and TgMIC8 are also shed during invasion (Brecht, Carruthers et al. 2001; Meissner, Reiss et al. 2002).

The rhoptry organelles are club shaped and secrete their contents during invasion as well. Rhoptry proteins play a critical role in formation of the tight junction (Alexander, Mital et al. 2005; Lebrun, Michelin et al. 2005), during invasion (Dubremetz 2007), and in host-parasite interaction (Saeij, Boyle et al. 2006; Taylor, Barragan et al. 2006), some rhoptry proteins are incorporated into the PVM after invasion (Beckers, Dubremetz et al. 1994). The rhoptry proteins also undergo proteolytic cleavage for proper function and targeting (Soldati, Lassen et al. 1998). The rhoptry proteins include many different kinases, proteases and phosphatases that may play a crucial role during invasion (Saeij, Boyle et al. 2006; Taylor, Barragan et al. 2006; Gilbert, Ravindran et al. 2007). Recently, it was shown that ROP 16 and ROP 18 are important for virulence of the parasite (Saeij, Boyle et al. 2006; Taylor, Barragan et al. 2006).

Another type of secretory organelle called the dense granules are present in the parasite. They are spread throughout the parasite and are exocytosed by the tachyzoites when they are inside the PV (Leriche and Dubremetz 1990). Some of the dense granule proteins are secreted constitutively and thought to play a role in host immune response (Vercammen, Scorza et al. 2000) and in modifying the parasitophorous vacuole (Leriche and Dubremetz 1990; Achbarou, Mercereau-Puijalon et al. 1991; Lecordier, Mercier et al. 1993; Lecordier, Moleon-Borodowsky et al. 1995; Cesbron-Delauw, Lecordier et al. 1996; Vercammen, Scorza et al. 2000) .

The periphery of the parasite consists of a plasma membrane and the two membranes of the inner membrane complex (IMC), a continuous layer of flattened vesicles underlying the plasma membrane (Mann and Beckers 2001). Below the IMC, there are 22 subpellicular microtubules, which run three fourths the length of the parasite. However, the IMC is found along the whole length of the parasite (Morrissette, Murray et al. 1997).

The pathology of *T. gondii* infections is thought to be due to the host immune response and lesions caused by host cell lysis. *T. gondii* invades almost any nucleated cell, replicates within the host cells and lyses out causing lesions. The process of *T. gondii* invasion is quite complex and parasite driven. There are two main steps during invasion, attachment and penetration. The parasite glides over the host cell surface by a process known as gliding motility. This type of motility does not involve any cell extensions or morphological changes. It is powered by an actin-myosin based motor complex known as the glideosome. The glideosome is a complex of myosin and three other proteins known as GAP45, GAP50 and the myosin light chain. (Opitz and Soldati 2002). The glideosome is anchored in the IMC by GAP50, which is an integral membrane glycoprotein (Gaskins, Gilk et al. 2004). Most proteins involved in gliding motility and in the invasion machinery are highly conserved within the apicomplexans (Opitz and Soldati 2002; Keeley and Soldati 2004; Baum, Richard et al. 2006). Upon some unknown recognition event, the parasite attaches to the host cell. The secretory organelles secrete their contents sequentially namely, micronemes, rhoptries and then dense granules (Carruthers and Sibley 1997). Microneme proteins MIC2-4 form the

bridge between the glideosome and the substrate. As the parasite invades the host cell, a moving junction forms at the point of entry. The moving junction is evident as a constriction of the host plasma membrane around the parasite (Aikawa, Komata et al. 1977; Alexander, Mital et al. 2005). As the parasite invades, the moving junction moves from the anterior to the posterior of the parasite. The apical membrane antigen 1 (AMA1), secreted from the micronemes, is associated with at least two other rhoptry proteins at the moving junction (Alexander, Mital et al. 2005; Lebrun, Michelin et al. 2005) . During invasion the parasite becomes surrounded by the PVM, made primarily of host cell plasma membrane (Suss-Toby, Zimmerberg et al. 1996).

After invasion is complete, the parasite resides and replicates within the PV. When the vacuole is filled with approximately 128 parasites, the parasites lyse open the host cell and egress to reinvade neighboring host cells. Parasites treated with cytochalasin D were unable to egress, suggesting active motility is required during the process (Black, Arrizabalaga et al. 2000; Moudy, Manning et al. 2001). It was earlier thought that parasites just rupture the host cells during egress. However, it has been shown that a moving junction is formed, similar to the one during invasion, and the parasites actively egress out of the host cell (Black and Boothroyd 2000; Hoff and Carruthers 2002; Arrizabalaga and Boothroyd 2004). Increases in intracellular calcium induce egress (Black, Arrizabalaga et al. 2000).

As mentioned earlier, *T. gondii* is a good model to study invasion by apicomplexans. In our lab, we are interested in the process of invasion of *T. gondii* into host cells. Using a novel small molecule approach we are trying to identify important

proteins and pathways involved in invasion. One of these proteins or pathways could eventually prove to be a good drug target for treatment of toxoplasmosis.

Background of small molecule screen

Invasion is one of the important aspects of the pathogenesis of *T. gondii* infection. A better understanding of the molecular mechanisms of invasion will be useful in developing drugs and vaccines for toxoplasmosis. *T. gondii* is a haploid organism, thus using forward genetic methods to study invasion is problematic. Disrupting an essential gene for invasion could prove lethal to the parasite and thus recovering mutants of interest will be challenging. Generating conditional mutants have alleviated this problem (Pfefferkorn and Pfefferkorn 1976; Meissner, Brecht et al. 2001; Mital, Meissner et al. 2005; White, Jerome et al. 2005; Mazumdar, E et al. 2006). However, it is not feasible to generate a large number of conditional mutants. An alternate approach to study invasion is to screen a library of structurally diverse small molecules for their effect on invasion. The small molecule approach has been a very useful tool for studying a variety of cell biological phenomena. For example, small molecules have been useful in identifying and characterizing proteins and functions of the eukaryotic cytoskeleton (Peterson and Mitchison 2002).

Our lab screened 12,160 structurally diverse small molecules using a high throughput invasion assay (Carey, Westwood et al. 2004). 24 inhibitors of invasion were identified from this screen. Unexpectedly, six enhancers of invasion were identified as well. Chapter 2 of this thesis was undertaken to test the hypothesis that one of the

inhibitors identified in this screen, 118793, is an inhibitor of cGMP phosphodiesterase (cGMP PDE). In the work described in Chapter 3, I tested whether the inhibitors of invasion also inhibit egress.

References

Achbarou, A., O. Mercereau-Puijalon, et al. (1991). "Differential targeting of dense granule proteins in the parasitophorous vacuole of *Toxoplasma gondii*." Parasitology 103 Pt 3: 321-9.

Aikawa, M., Y. Komata, et al. (1977). "Transmission and scanning electron microscopy of host cell entry by *Toxoplasma gondii*." Am J Pathol 87(2): 285-96.

Alexander, D. L., J. Mital, et al. (2005). "Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles." PLoS Pathog 1(2): e17.

Arrizabalaga, G. and J. C. Boothroyd (2004). "Role of calcium during *Toxoplasma gondii* invasion and egress." Int J Parasitol 34(3): 361-8.

Baum, J., D. Richard, et al. (2006). "A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites." J Biol Chem **281**(8): 5197-208.

Beckers, C. J., J. F. Dubremetz, et al. (1994). "The *Toxoplasma gondii* rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm." J Cell Biol 127(4): 947-61.

Black, M. W., G. Arrizabalaga, et al. (2000). "Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress." Mol Cell Biol 20(24): 9399-408.

Black, M. W. and J. C. Boothroyd (2000). "Lytic cycle of *Toxoplasma gondii*." Microbiol Mol Biol Rev 64(3): 607-23.

Brecht, S., V. B. Carruthers, et al. (2001). "The *Toxoplasma* micronemal protein MIC4 is an adhesin composed of six conserved apple domains." J Biol Chem 276(6): 4119-27.

Brossier, F., T. J. Jewett, et al. (2005). "A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by *Toxoplasma*." Proc Natl Acad Sci U S A 102(11): 4146-51.

Brydges, S. D., G. D. Sherman, et al. (2000). "Molecular characterization of TgMIC5, a proteolytically processed antigen secreted from the micronemes of *Toxoplasma gondii*." Mol Biochem Parasitol 111(1): 51-66.

Brydges, S. D., X. W. Zhou, et al. (2006). "Targeted deletion of MIC5 enhances trimming proteolysis of *Toxoplasma* invasion proteins." Eukaryot Cell 5(12): 2174-83.

Carey, K. L., N. J. Westwood, et al. (2004). "A small-molecule approach to studying invasive mechanisms of *Toxoplasma gondii*." Proc Natl Acad Sci U S A 101(19): 7433-8.

Carruthers, V. B., S. N. Moreno, et al. (1999). "Ethanol and acetaldehyde elevate intracellular [Ca2+] and stimulate microneme discharge in *Toxoplasma gondii*." Biochem J 342 (Pt 2): 379-86.

Carruthers, V. B. and L. D. Sibley (1997). "Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts." Eur J Cell Biol 73(2): 114-23.

Carruthers, V. B. and L. D. Sibley (1999). "Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*." Mol Microbiol 31(2): 421-8.

Cesbron-Delauw, M. F., L. Lecordier, et al. (1996). "Role of secretory dense granule organelles in the pathogenesis of toxoplasmosis." Curr Top Microbiol Immunol 219: 59-65.

Dowse, T. and D. Soldati (2004). "Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis." Curr Opin Microbiol 7(4): 388-96.

Dowse, T. J., J. C. Pascall, et al. (2005). "Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion." Int J Parasitol 35(7): 747-56.

Dubremetz, J. F. (2007). "Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction." Cell Microbiol 9(4): 841-8.

Fourmaux, M. N., A. Achbarou, et al. (1996). "The MIC1 microneme protein of *Toxoplasma gondii* contains a duplicated receptor-like domain and binds to host cell surface." Mol Biochem Parasitol 83(2): 201-10.

Gaskins, E., S. Gilk, et al. (2004). "Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*." J Cell Biol 165(3): 383-93.

Gilbert, L. A., S. Ravindran, et al. (2007). "*Toxoplasma gondii* targets a protein phosphatase 2C to the nuclei of infected host cells." Eukaryot Cell 6(1): 73-83.

Hoff, E. F. and V. B. Carruthers (2002). "Is *Toxoplasma egress* the first step in invasion?" Trends Parasitol 18(6): 251-5.

Hu, K., D. S. Roos, et al. (2002). "A novel polymer of tubulin forms the conoid of *Toxoplasma gondii*." J Cell Biol 156(6): 1039-50.

Keeley, A. and D. Soldati (2004). "The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa." Trends Cell Biol 14(10): 528-32.

Kim, K. and L. M. Weiss (2004). "*Toxoplasma gondii*: the model apicomplexan." Int J Parasitol 34(3): 423-32.

Lebrun, M., A. Michelin, et al. (2005). "The rhoptry neck protein RON4 re-localizes at the moving junction during *Toxoplasma gondii* invasion." Cell Microbiol 7(12): 1823-33.

Lecordier, L., C. Mercier, et al. (1993). "Molecular structure of a *Toxoplasma gondii* dense granule antigen (GRA 5) associated with the parasitophorous vacuole membrane." Mol Biochem Parasitol 59(1): 143-53.

Lecordier, L., I. Moleon-Borodowsky, et al. (1995). "Characterization of a dense granule antigen of *Toxoplasma gondii* (GRA6) associated to the network of the parasitophorous vacuole." Mol Biochem Parasitol 70(1-2): 85-94.

Leriche, M. A. and J. F. Dubremetz (1990). "Exocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion." Parasitol Res 76(7): 559-62.

Lovett, J. L., N. Marchesini, et al. (2002). "*Toxoplasma gondii* microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores." J Biol Chem 277(29): 25870-6.

Lovett, J. L. and L. D. Sibley (2003). "Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells." J Cell Sci 116(Pt 14): 3009-16.

Mann, T. and C. Beckers (2001). "Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite *Toxoplasma gondii*." Mol Biochem Parasitol 115(2): 257-68.

Meissner, M., S. Brecht, et al. (2001). "Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*." Nucleic Acids Res 29(22): E115.

Meissner, M., M. Reiss, et al. (2002). "A family of transmembrane microneme proteins of *Toxoplasma gondii* contain EGF-like domains and function as escorters." J Cell Sci 115(Pt 3): 563-74.

Mital, J., M. Meissner, et al. (2005). "Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion." Mol Biol Cell 16(9): 4341-9.

Mondragon, R. and E. Frixione (1996). "Ca(2+)-dependence of conoid extrusion in *Toxoplasma gondii* tachyzoites." J Eukaryot Microbiol 43(2): 120-7.

Monteiro, V. G., E. J. de Melo, et al. (2001). "Morphological changes during conoid extrusion in Toxoplasma gondii tachyzoites treated with calcium ionophore." J Struct Biol 136(3): 181-9.

Morrissette, N. S., J. M. Murray, et al. (1997). "Subpellicular microtubules associate with an intramembranous particle lattice in the protozoan parasite *Toxoplasma gondii*." J Cell Sci 110(Pt 1): 35-42.

Moudy, R., T. J. Manning, et al. (2001). "The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*." J Biol Chem 276(44): 41492-501.

Opitz, C., M. Di Cristina, et al. (2002). "Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite *Toxoplasma gondii*." EMBO J 21(7): 1577-85.

Opitz, C. and D. Soldati (2002). "'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*." Mol Microbiol 45(3): 597-604.

Peterson, J. R. and T. J. Mitchison (2002). "Small molecules, big impact. A history of chemical inhibitors and the cytoskeleton." Chem Biol 9(12): 1275-85.

Pfefferkorn, E. R. and L. C. Pfefferkorn (1976). "*Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants." Exp Parasitol **39**(3): 365-76.

Roos, D. S., M. J. Crawford, et al. (1999). "Transport and trafficking: Toxoplasma as a model for Plasmodium." Novartis Found Symp 226: 176-95; discussion 195-8.

Saeij, J. P., J. P. Boyle, et al. (2006). "Polymorphic secreted kinases are key virulence factors in toxoplasmosis." Science 314(5806): 1780-3.

Soldati, D., A. Lassen, et al. (1998). "Processing of *Toxoplasma* ROP1 protein in nascent rhoptries [In Process Citation]." Mol Biochem Parasitol 96(1-2): 37-48.

Suss-Toby, E., J. Zimmerberg, et al. (1996). "*Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore." Proc Natl Acad Sci U S A **93**(16): 8413-8.

Taylor, S., A. Barragan, et al. (2006). "A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*." Science 314(5806): 1776-80.

Teo, C. F., X. W. Zhou, et al. (2007). "Cysteine protease inhibitors block Toxoplasma gondii microneme secretion and cell invasion." Antimicrob Agents Chemother 51(2): 679-88.

Vercammen, M., T. Scorza, et al. (2000). "DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice." Infect Immun 68(1): 38-45.

Wan, K. L., V. B. Carruthers, et al. (1997). "Molecular characterisation of an expressed sequence tag locus of *Toxoplasma gondii* encoding the micronemal protein MIC2." Mol Biochem Parasitol 84(2): 203-14.

Yap, G. S. and A. Sher (1999). "Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function." Immunobiology 201(2): 240-7.

CHAPTER II

Target identification of 118793

Introduction

From the high throughput screen described in chapter 1, 24 inhibitors of *T. gondii* invasion were identified (Carey, Westwood et al. 2004). One of the inhibitors, 118793 (Fig II-1), is structurally identical to monastroline (HR22C16), an inhibitor of the mammalian kinesin Eg5 (Hotha, Yarrow et al. 2003). 118793 is also structurally similar to Tadalafil (Cialis), an inhibitor of mammalian cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) (Daugan and Labaudiniere 2000; Daugan, Grondin et al. 2003; Daugan, Grondin et al. 2003).

118793 is an irreversible inhibitor of invasion. 118793 inhibits invasion at concentrations as low as 12.5 μM. It inhibits motility, at concentrations as low as 25 μM. It inhibits both constitutive and induced microneme secretion. 118793 targets the parasite and not the host cell during invasion (Carey, Westwood et al. 2004).

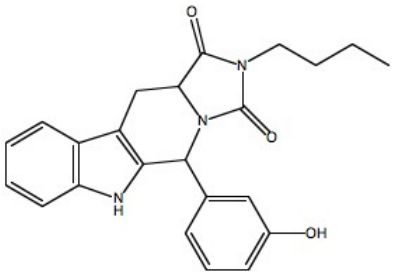

Figure II- 1 Structure of 118793

Although 118793 is structurally identical to monastroline, from the bioinformatics work and structural analysis studies described below, kinesins seem an unlikely target.

Bioinformatic analysis of *Toxoplasma gondii* kinesins

Keyword search for the word “Kinesin” in ToxoDB identified 19 protein/genes as kinesins. However, after performing a BLASTP search, only 15 of those proteins/genes contain a predicted kinesin motor domain.

Of the 15 proteins, eight are expressed in tachyzoites, two in bradyzoites and two in oocysts. No ESTs were identified for four of the 15 protein/genes. Eg5 belongs to the family 5 kinesins in mammals. Comparison of the conserved regions of different families of kinesins was performed using BLASTP. Four of the 15 proteins/genes had similarity to family 5 kinesin, and at least three out of the four are expressed in tachyzoites.

Through hydrogen/deuterium exchange mass spectrometry, two regions forming the inhibitor-binding pocket of Eg5 where identified (Brier, Lemaire et al. 2006). Comparing the sequence alignments of these two regions and the four family 5-like genes, only two out of the four genes had similarities in region I. All four genes had similarities in region II. However, since we do not know which of the residues are required for inhibitor binding, it is hard to tell if 118793 interacts with these four proteins in *T. gondii*. A collection of 480 inhibitors with known targets were tested in our invasion assay (unpublished data). None of the known kinesin inhibitors in this collection inhibited *T. gondii* invasion. Furthermore, Eg5 is involved in mitosis in mammals. Even

if the Eg5 like kinesins were involved in mitosis in *T. gondii*, no mitosis occurs within the 20-30 second window of *T. gondii* invasion. These data suggest that kinesins may not be the target of the 118793 during invasion.

cGMP and cGMP PDE

The goal of my project was to test if cGMP PDE was a target of 118793 during invasion. cGMP is a second messenger molecule produced by an enzyme called guanylate cyclase. Two types of guanylate cyclases are found in most cells: particulate guanylate cyclases and soluble guanylate cyclases. Peptide ligands and nitric oxide stimulate the production of cGMP by particulate gunaylate cyclase and soluble guanylate cyclase respectively (Zhao, Brandish et al. 1999; Davies 2006). Some of the known downstream targets of cGMP are cyclic nucleotide gated channels (CNGs), cGMP-regulated phosphodiesterases (cGMP-PDEs) and cGMP-dependent protein kinases (PKGs) (Davies 2006).

When cGMP binds to cyclic nucleotide-gated (CNG) channels it opens the channels, which brings about a change in concentrations of various ions, especially calcium (Davies 2006). Ca2+/calmodulin and phosphorylation regulate the activity of CNG channels. CNG channels are found in many different type of cells, but predominantly in neurons (Kaupp and Seifert 2002). However, the exact function of cyclic nucleotide-gated (CNG) channels is known only in in retinal photoreceptors (Yau and Baylor 1989) and in olfactory epithelium (Nakamura T 1987).

The cGMP dependent protein kinases (PKG) are serine/threonine kinases found in many different eukaryotic organisms and cells (Francis and Corbin 1999;

Hofmann, Feil et al. 2006). PKG is a soluble enzyme, and enriched in cells like smooth muscle cells, neuroblastoma and glioma hybrid cells and Purkinje cells. A single gene encodes both the catalytic and regulatory domains (containing cGMP-binding sites) of PKG, in contrast to different genes encoding the two domains of PKA (cAMP-dependent protein kinase) (Francis and Corbin 1999).

In the absence of cGMP, the N terminus of PKG interacts with the catalytic domain. Thus, the protein is inactive. When cGMP is present, it binds to the regulatory domain, which in turn causes a conformational change. This results in the release of the N terminus from the catalytic domain. Activated PKG can then phosphorylate its substrates such as myosin light chain phosphatase in smooth muscle cells. Phosphorylated myosin light chain phosphatase in turn dephosphorylates the myosin light chain resulting in muscle relaxation (Wikipedia)(Wu, Haystead et al. 1998).

All PKG sequences known so far contain two cGMP-binding sites, however, apicomplexans PKGs have three cGMP-binding sites (Deng, Parbhu-Patel et al. 2003). Apicomplexan parasite PKGs are specific drug targets. The inhibitor, known as compound 1 (a trisubstituted pyrrole, 4-[2-(fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl]pyridine), was active against a number of apicomplexan parasites. It has been demonstrated conclusively that the parasite PKG is the primary target of compound 1 (Donald, Allocco et al. 2002). PKG could only be disrupted in *T. gondii*, which is a haploid organism, in the presence of an episomal copy of the gene. This suggested that PKG is an essential protein in apicomplexans.

E. tenella and *T. gondii* mutant PKGs with greatly reduced binding affinity for compound 1 were generated by site directed mutagenesis. This provided further evidence that PKG is the primary target for compound 1. It has now been shown that compound 1 prevents attachment of *E. tenella* sporozoites and *T. gondii* tachyzoites to host cells, but also inhibits gliding motility and cell invasion (Wiersma, Galuska et al. 2004). Inhibition of gliding motility and cell invasion by compound 1 did not occur in parasites expressing compound 1-insensitive PKG mutants, implying an important role for PKG in these events.

The intracellular concentration and duration of cGMP levels is regulated by the activity of phosphodiesterases (PDE). More than one type of PDE can be found in a single cell. cGMP levels are modulated by the activity of specific PDEs and the localization of the PDEs in the cell. On the other hand, the activity of PDE can be regulated by cGMP, phosphorylation, dephosphorylation, $Ca2^{+}$/ calmodulin, and interactions with other proteins (Bender and Beavo 2006). There are 11 families of mammalian PDEs (Bender and Beavo 2006). All PDEs have a catalytic domain and a regulatory domain. All 11 families are highly conserved in the catalytic domain. Of the 11 families PDE 1, 2, 5, 6 and to a lesser extent 10 and 11 are cyclic nucleotide PDEs. These enzymes have two cGMP binding sites, known as GAF A and GAF B domains, in their regulatory domain. Binding of cGMP regulates their activity because they are conformationally sensitive to cGMP (Bender and Beavo 2006).

PDE 1 is a calcium/ calmodulin dependent PDE that hydrolyses both cAMP and cGMP. The specificity for the two nucleotides is different for the different isoforms

of the PDE 1 family enzymes. PDE 2 family enzymes also hydrolyse cAMP and cGMP. Binding of cGMP to the GAF B domain regulates the activity of PDE 2 enzymes. PDE 3 family members hydrolyse both cGMP and cAMP. However, the cAMP hydrolyzing activity is inhibited in the presence of cGMP. Thus PDE 3 family is also the cGMP inhibited PDE family. PDE 5 family members also have the GAF domains and are thus regulated by the binding of cGMP. However, in contrary to PDE 2 cGMP binds to the GAF A domain of PDE 5 enzymes. PDE 6 is known as the photoreceptor phosphodiesterase. It is predominantly expressed in the photoreceptor outer segment of the retina. PDE 10 also contain the GAF domain, however they bind cAMP and not cGMP. PDE 11 hydrolyzes both cAMP and cGMP. However, it contains the GAF domains which are regulated by cGMP binding (Bender and Beavo 2006).

There are many different isoforms of PDE and each has a distinct architecture at the active site. This makes them a very good therapeutic target for various diseases and conditions. For example, Sildenafil, Tadalafil and Vardenafil are clinically used inhibitors of PDE 5 to treat erectile dysfunction. All three drugs bind to the catalytic site of PDE 5. The residues and domains they bind to make them very specific PDE 5 inhibitors. Rolipram is a specific inhibitor of PDE 4.

As mentioned earlier, 118793 is structurally similar to Tadalafil. From the bioinformatics work described below, there are 16 PDE domain-containing protein/genes in *T. gondii*. However, none have any homology to mammalian PDE 5. I continued to test if cGMP PDE is a target of 118793 because there was some preliminary data supporting this hypothesis (Kim Carey and Gary Ward, unpublished data). PDE Assays were

performed using a fluorescent substrate and purified bovine PDE. In these assay 118793 appeared to inhibit purified PDE. In addition, this hypothesis is supported by studies implicating cGMP and cGMP dependent protein kinase (PKG) in invasion (Wiersma, Galuska et al. 2004; Donald, Zhong et al. 2006). Using an inhibitor of PKG, Compound 1, it was shown that PKG is required for motility and invasion (Gurnett, Liberator et al. 2002).

Bioinformatic analysis of cGMP PDEs

A keyword search for the word "Phosphodiesterase" in ToxoDB identified 21 protein/genes as putative PDEs. Of these, only 16 (gene identification numbers listed below) contain the PDE conserved catalytic domain.

20.m03727	44.m02764	57.m03117	72.m00380
641.m01498	83.m01190	39.m00361	44.m02765
583.m05366	42.m03630	42.m03528	42.m03383
49.m03267	59.m03644	55.m04764	55.m00270

When I looked for ESTs in ToxoDB, 5 are expressed in tachyzoites, 4 are expressed in both tachyzoites and bradyzoites, 1 in oocysts and 6 are non-stage specific.

Tachyzoite ESTs	Bradyzoite ESTs	Oocyst ESTs	Unclear (no ESTs)
20.m03727	49.m03267	55.m04764	39.m00361
42.m03383	57.m03117		42.m03630
42.m03528	83.m01190		44.m02764
49.m03267	72.m00380		44.m02765
55.m00270			59.m03644

Tachyzoite ESTs	Bradyzoite ESTs	Oocyst ESTs	Unclear (no ESTs)
57.m03117			641.m01498
583.m05366			
83.m01190			
72.m00380			

Experimental approach

I approached the question if 118793 inhibits cGMP PDE during invasion both *in vivo* and *in vitro*. I performed *in vitro* PDE assays, in the presence and absence of 118793 using fluorescent and radiolabeled substrates. I eventually abandoned the fluorescent substrate-based assay, as the assay gave variable results. The variability was compounded by the fact that 118793 is also fluorescent at the same wavelengths as the substrate. I have described the approach using radiolabeled substrate below. I also generated a fluorescent cGMP indicator to test the hypothesis *in vivo* as described below.

II.A *In vitro* PDE assay using radiolabeled substrate

Principle

^{3}[H] cGMP is hydrolyzed to the radiolabeled 5' nucleotide product ^{3}[H] GMP, by PDE (parasite extract). GMP is then hydrolyzed to the corresponding nucleoside, ^{3}[H] guanosine, using snake venom (nucleotidase) (Figure II-2). cGMP and GMP are negatively charged and bind to an anion exchange column. Guanosine is

uncharged and will flow through when eluted with water. I measured the radioactivity in the flow-through, which is a measure of the PDE activity in the parasite extract.

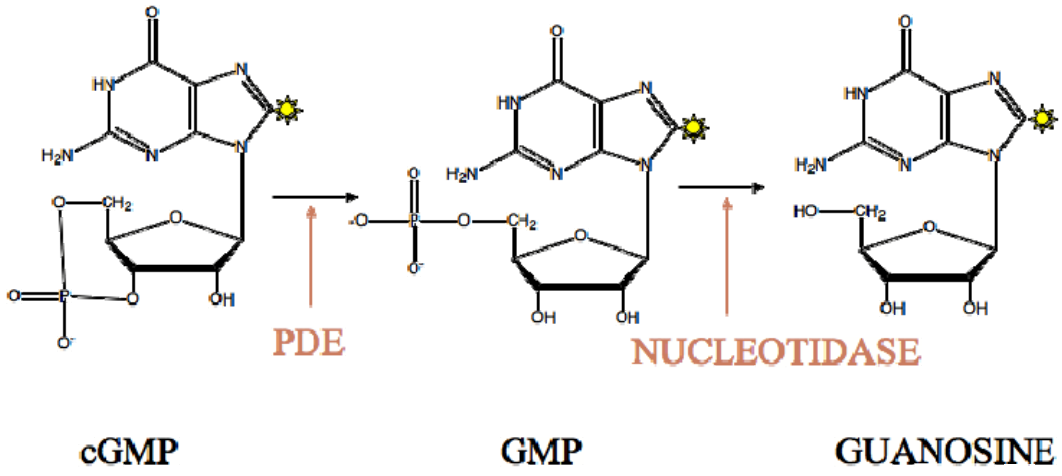

Figure II- 2 Conversion of ^{3}H- cGMP to ^{3}H- guanosine in the cGMP PDE assay

Method

The assay was performed as previously described (Thompson, Brooker et al. 1974). The enzyme diluent (50 mM Hepes at pH 7.2, 0.5 mg/ml ovalbumin and 0.1 mg/ml protease inhibitor (Sigma)) and reaction mix (500 µM tritiated cGMP, 0.5 µM cold cGMP, 1 mM magnesium chloride, 0.2 mg/ml ovalbumin, 0.1 mM EDTA and 50 mM Hepes, pH 7.4) were prepared as described (Thompson, Brooker et al. 1974). Purified bovine heart PDE (dissolved in 50 % glycerol to final concentration of 0.005 units /µl) and snake venom were purchased from Sigma. Parasites were harvested and extracted in Tris-buffer (0.1 M Tris-HCl, 20 mM magnesium chloride and 10 mM calcium chloride) with 0.5 % Triton X-100 and protease inhibitors. To prepare the anion exchange column; 100 ml of the Sephadex resin was precycled with water (2X), 0.1 N

hydrochloric acid (1X), water (2X), 0.1 N sodium hydroxide (1X) , water (2X), 0.1 N hydrochloric acid (1X) and water (4X). pH was adjusted to 7 with 50 mM Hepes at pH 8. Disposable polypropylene columns (Biorad) were packed with 4ml resin before use.

For the PDE assay, the enzyme diluent (60 μl), an appropriate amount of enzyme or parasite extract (final volume 40 μl) and reaction mix (200 μl) were mixed on ice. The total reaction was incubated at 30˚C for 30min. The reaction was stopped by adding 100 μl 0.25 M hydrochloric acid and neutralized with 0.25 M sodium hydroxide. To the reaction, 100 μl of snake venom was added and incubated at 30˚C for 30 min. The total reaction was added to the columns and eluted with 8 mls of water. 1 ml of the eluate was mixed with 10 ml of scintillation fluid and counted. 118793 (from a 40 mM stock) or equivalent volumes of DMSO were added to the reaction just before adding the reaction mix.

Results

(i) I have tested and seen activity with 2 x 10 ^{7} to 2 x 10 ^{8} tachyzoites. The following graph represents an assay with increasing amounts of parasite extract, done in duplicate (Figure II-3). As can be seen, the activity measured is roughly proportional to the amount of extract added.

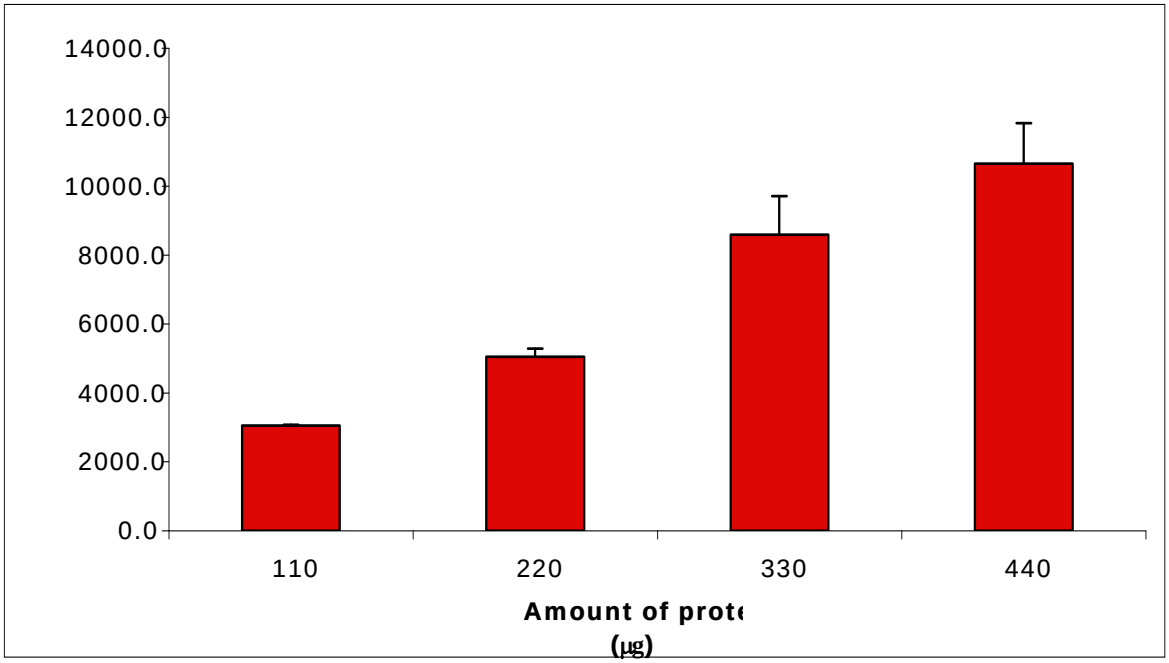

Figure II- 3 PDE assay with parasite extracts

(ii) I then tested whether 118793 inhibits PDE activity in the parasite extracts (Figure II-4). I pretreated 5 x 10^{7} parasites with 100 μM 118793, IBMX (positive control) or DMSO for 15min, extracted the parasites and did a 30min assay. The PDE control is untreated parasite extract. From this assay it did not seem like parasite PDE was inhibited by 118793. In fact, even the positive control IBMX does not seem to inhibit.

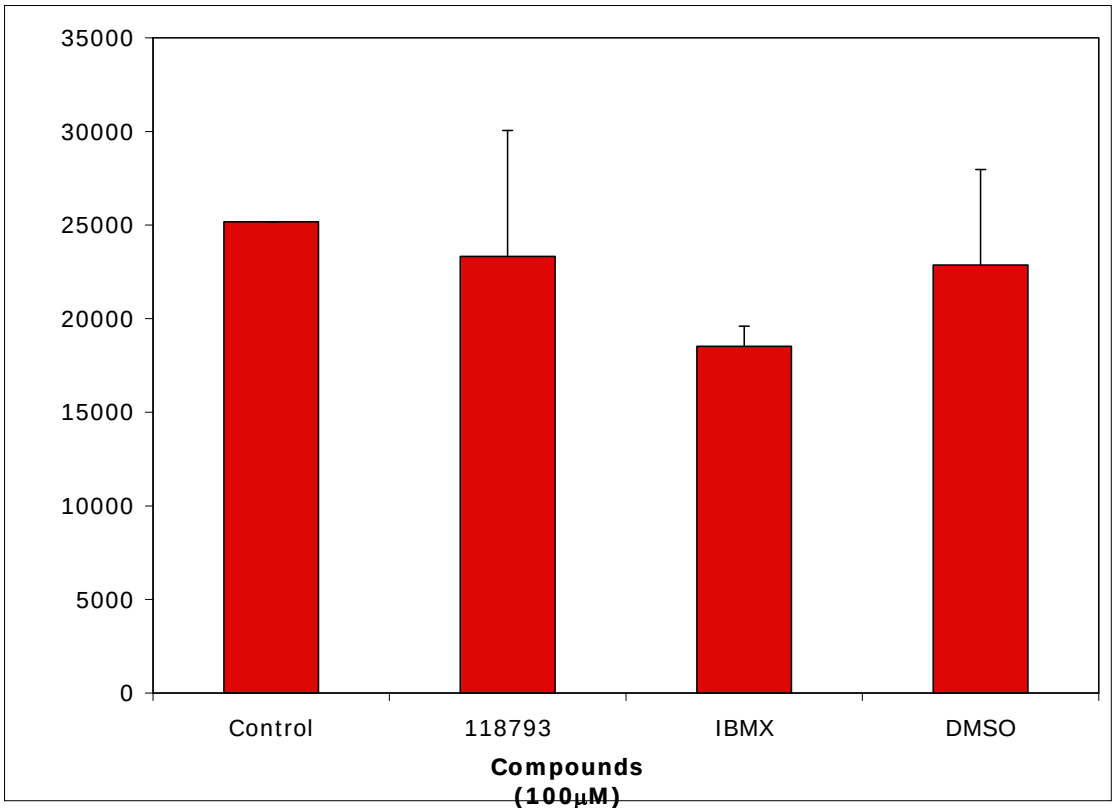

Figure II- 4 PDE assay with parasites extracts- compound added before extraction

(iii) I repeated the assay by adding the compounds directly to the extracts without pre-treatment (Figure II-5) . The compounds still do not seem to inhibit.

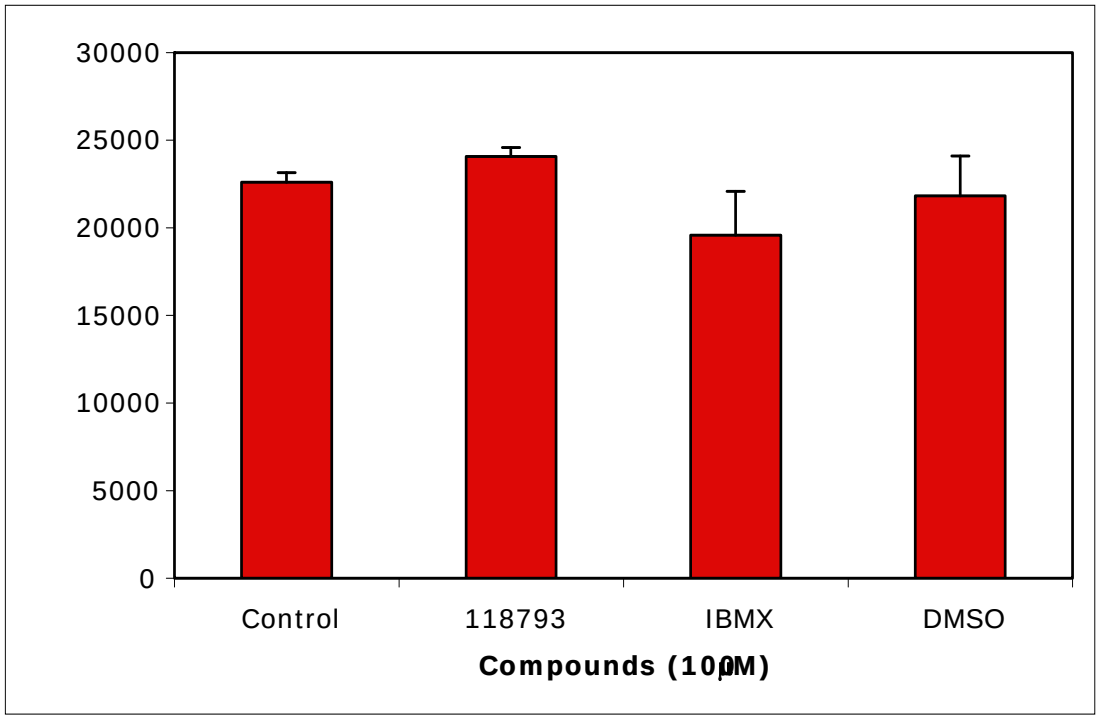

Figure II- 5 PDE assay with parasite extracts- compound added after extraction

(iv) I next tested different concentrations of IBMX (25μM to 300μM) (data not shown). I still saw no inhibition with IBMX. It is possible that IBMX does not inhibit PDE in *T. gondii*. The other possibility is that the activity we see is not actually due to PDE in the extract but some other activity that hydrolyzes cGMP.

(v) I repeated all assays with commercial bovine brain PDE (crude preparation; undefined isoforms) to make sure that the IBMX is active (Figure II-6). I first made sure that I could see activity with the store bought PDE in my assay. The following graph represents the direct correlation between increasing amounts of PDE and the measured activity of PDE.

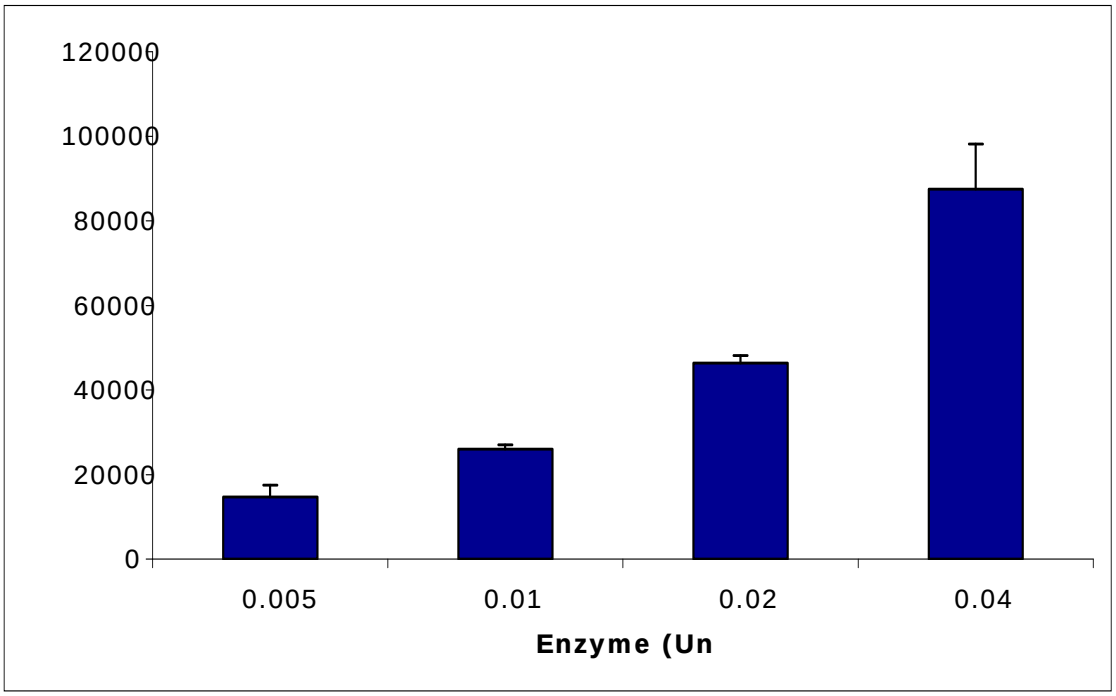

Figure II- 6 PDE assay with purified bovine PDE

vi) I then tested the effect of 118793 and IBMX on the bovine PDE, at 300 μM and 100 μM (Figure II-7). As seen from the graph below, IBMX inhibited approximately 50 % of PDE activity. However, 118793 did not inhibit activity even at 300μM.

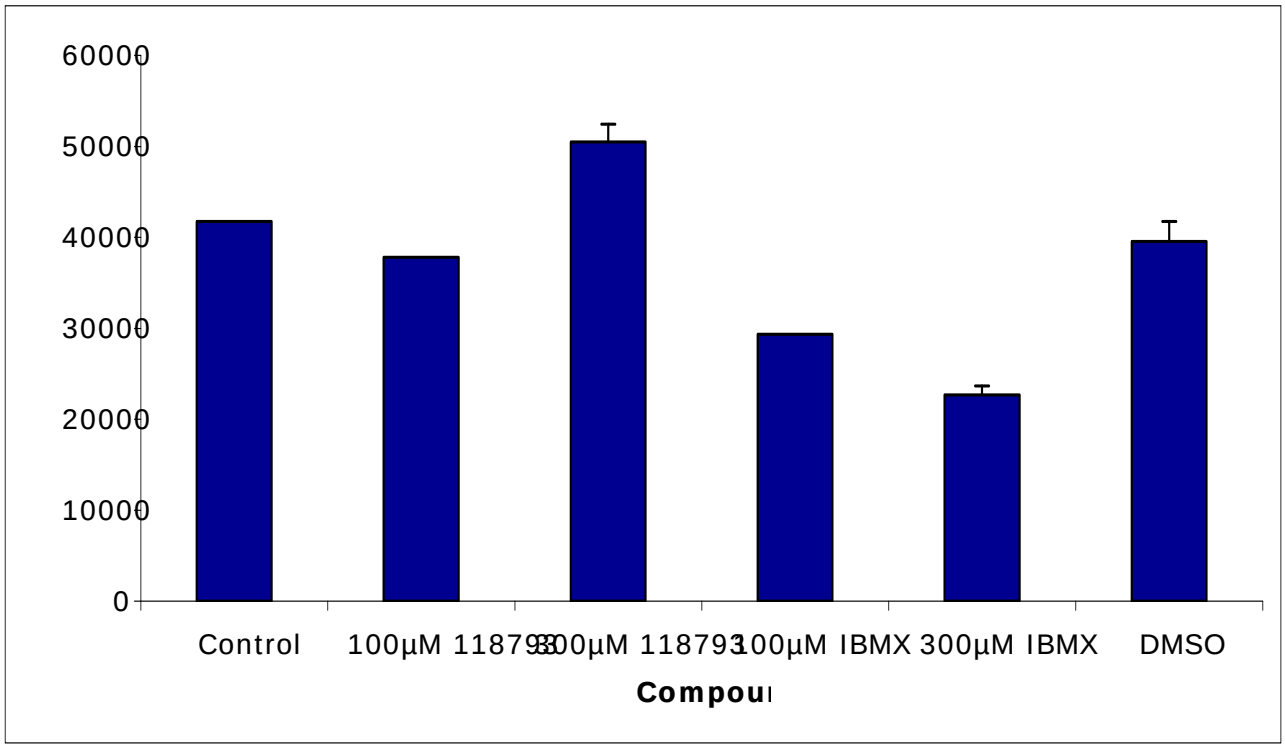

Figure II- 7 PDE assay with purified PDE in the presence of compounds

vii) When a dose response assay was done, I saw the same pattern (Figure II-8). IBMX inhibited bovine PDE activity in a dose dependent manner, while 118793 did not inhibit activity. The following graph shows the results of the assay done in duplicate.

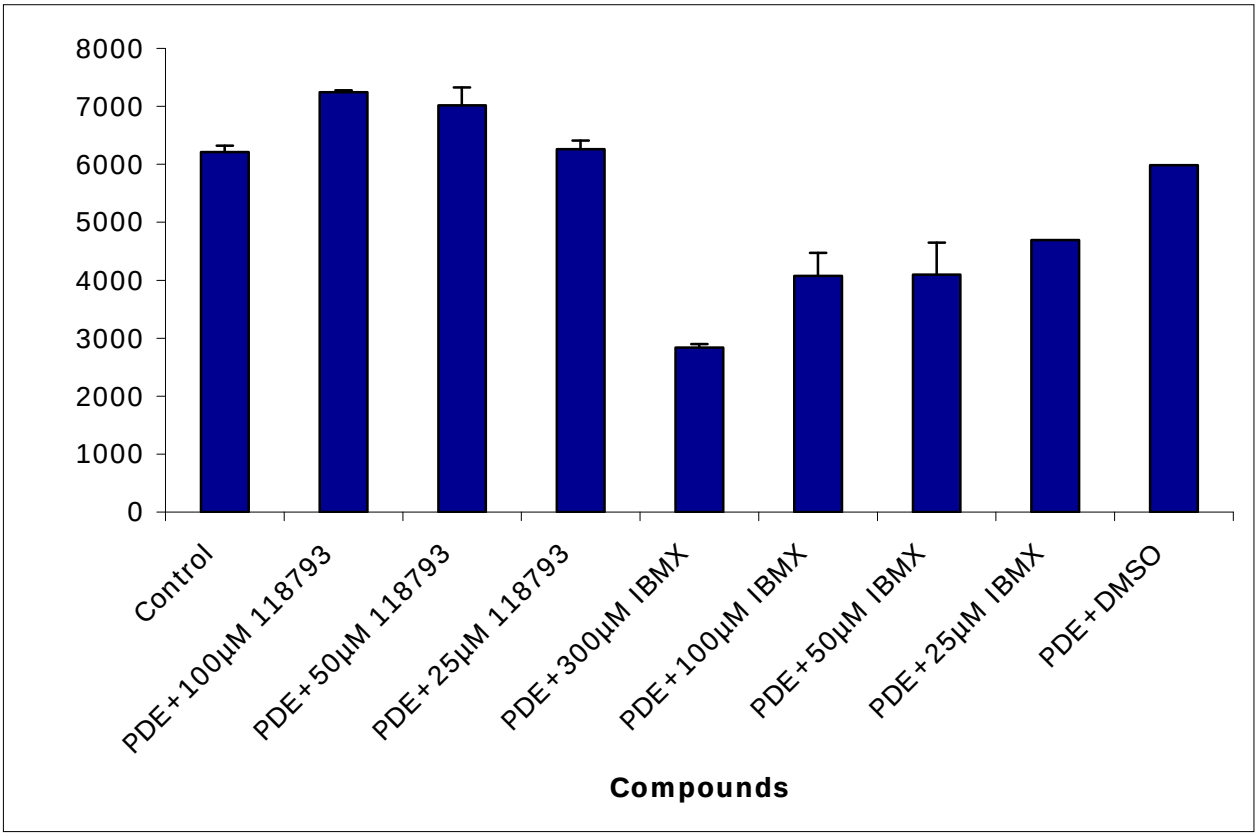

Figure II- 8 PDE assay with purified PDE in the presence of compounds at different concentrations

viii) From the above results it was clear that 118793 did not inhibit bovine PDE. IBMX inhibited up to 50% of bovine PDE activity, however it did not affect the activity in parasite extracts. It is possible that the level of PDE is very low in RH strain, so I decided to repeat the assay with PTG strain parasites (because RH and P strain parasites show differences in cyclic nucleotide metabolism (Kirkman, Weiss et al. 2001). As seen from the following graph (Figure II-9), there was no difference between RH and PTG parasites.

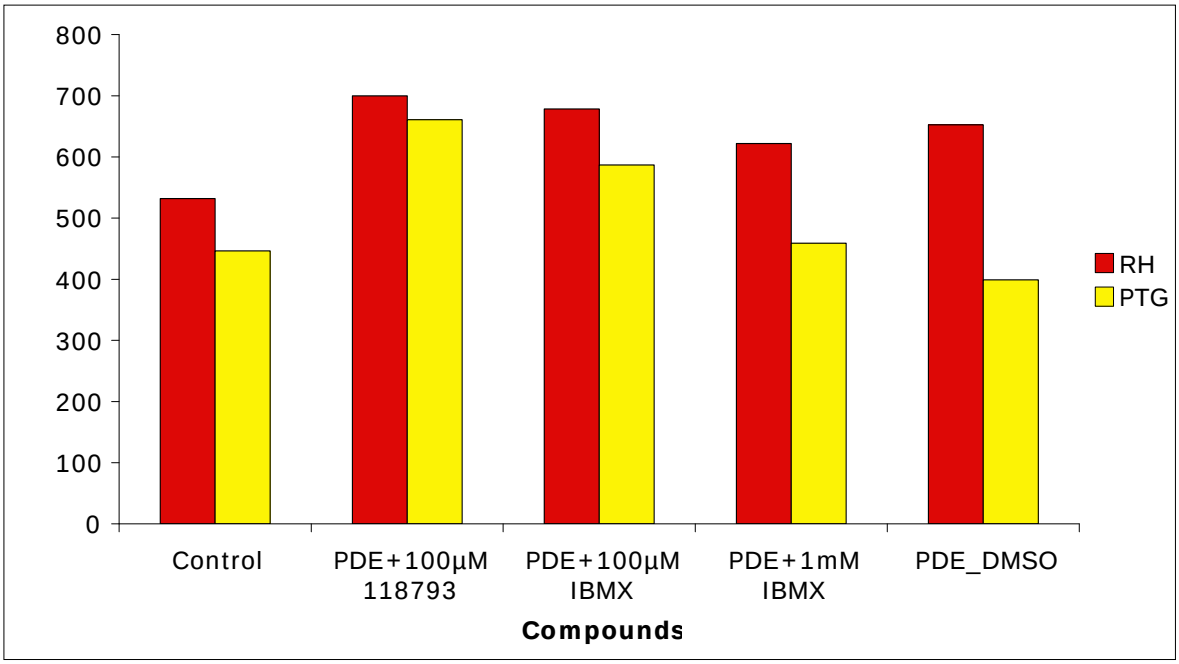

Figure II- 9 PDE assay with RH and PTG parasites extracted after treatment with compounds

ix) I then tested RH, PTG and purified PDE at the same time (Figure II-10). IBMX still inhibited purified PDE, but had no detectable effect on RH or PTG extracts.

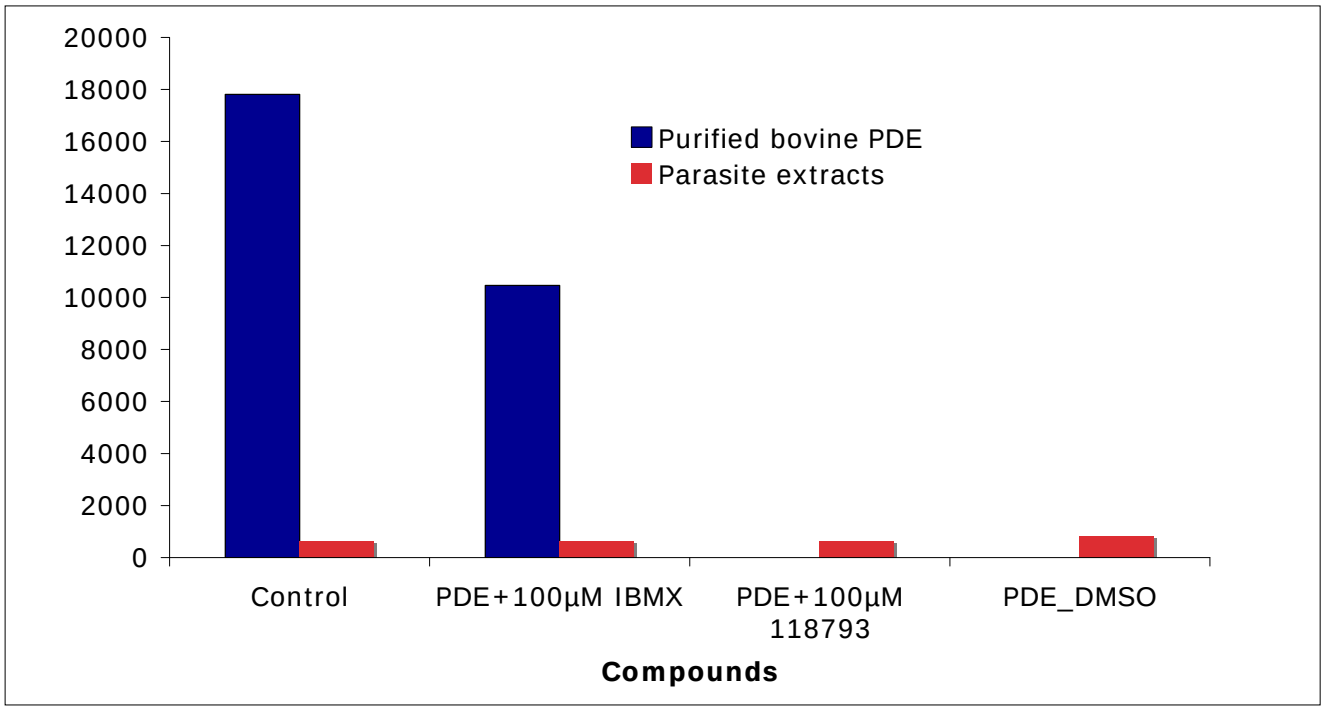

Figure II- 10 PDE assay with parasite extracts and purified PDE

Discussion

From the assays it is clear that there is no inhibition of PDE activity by IBMX in parasite extracts. There are four possible explanations for the results: A) The activity I see with parasite extracts may in fact not be due to PDE. It could be that the membrane and other cellular debris in the extracts caused the counts to flow through the ion exchange column; B) The level of PDE could be too low in the extracts to see any differences that exist; C) Parasite PDE is not inhibited by IBMX; D) Parasite PDE may be inactive or denatured during the preparation of the parasite extracts. Due to the lack of a positive control, it is hard to conclude if 118793 inhibits PDE in parasite extracts. However, IBMX inhibits 50% of purified PDE activity but 118793 does not inhibit purified PDE.

Future experiments could include spinning down the parasite extracts and repeating the experiments with the supernatant. This will clear the membranes and other debris, which may be causing counts to flow through the column non-specifically. To address the question if parasite PDE is inactivated during the extraction procedure, parasite extracts could be prepared by alternative methods. The extraction could be done using different detergents, homogenization or in different buffers. An alternative approach to the anion exchange method could be to detect the products of the assay using thin layer chromatography (Bohme and Schultz 1974).

Although 118793 has no effect on purified PDE, it is still worth pursuing the question as to whether parasite cGMP PDE is inhibited by 118793. The inhibitor of mammalian cGMP PDE, which is structurally similar to 118793, is a highly specific

inhibitor of PDE 5. However, it also inhibits other PDE isoenzymes to much lesser extent. PDE 6 is inhibited 10 fold less than PDE 5 and there are PDE 6-like PDEs in *T. gondii*. Another reason to pursue the question is there are four possible stereoisomers of 118793. Three are active and inhibit invasion while one is inactive. If 118793 inhibits parasite PDE, it will be interesting to see the correlation or lack thereof between the effects of the stereoisomers on invasion and PDE activity.

II.B Fluorescence resonance energy transfer (FRET) and CYGNET 2.1 (cyclic GMP indicators using energy transfer)

To address whether 118793 inhibits cGMP PDE *in vivo*, I tried to express a FRET indicator of cGMP in intact cells. CYGNET 2.1 (Honda, Sawyer et al. 2005) is a genetically encoded indicator of cGMP. Such indicators are very sensitive and are a useful tool to study signaling in different organisms. They are especially helpful in studying dynamic changes in levels of the signaling molecules. CYGNET 2.1 is used to measure the levels of cGMP in live cells. However, modified versions of these FRET based indicators have also been used successfully to study calcium (Honda, Moosmeier et al. 2005; Honda, Sawyer et al. 2005) and cAMP (Miyawaki, Llopis et al. 1997; Miyawaki, Griesbeck et al. 1999). CYGNET 2.1 is highly selective for cGMP, because the indicator contains PKG, which is conformationally sensitive to levels of cGMP. The binding of cGMP to PKG is reversible, making studies of dynamic interactions and changes of cGMP possible using CYGNET 2.1. CYGNET 2.1 has been useful in

studying changes in cGMP levels in single cells without affecting any other cell processes (Honda, Adams et al. 2001; Sawyer, Honda et al. 2003).

Principle

CYGNET 2.1 consists of a portion of the PKG protein flanked by CFP and Citrine. Residues 1-77 from the N-terminus of the PKG protein are deleted and Thr516 is mutated to alanine rendering it catalytically inactive (Figure II-11). There are many advantages of using PKG in the CYGNET 2.1 indicator. PKG is not restricted to membranes, it has a very high affinity for cGMP and it undergoes a conformational change in response to binding cGMP (Ruth, Landgraf et al. 1991; Zhao, Trewhella et al. 1997; Wall, Francis et al. 2003). The principle behind the CYGNET 2.1 indicator system is that when PKG is not bound to cGMP, the fluorophores CFP and Citrine are close enough to each other that FRET transfer can occur from CFP to Citrine. When CFP is excited at 440nm, it emits at 475nm. Citrine, when excited at 475nm, emits at 525nm. Using a dual emission florescence microscope, emission can be measured at both wavelengths and the FRET ratio of CFP/Citrine can be calculated. When cGMP is bound to PKG, PKG undergoes a conformational change, which moves CFP and Citrine further from each other. This decreases the energy transfer from CFP to Citrine and there is a decrease in CFP/Citrine FRET ratio (Figure II-12).

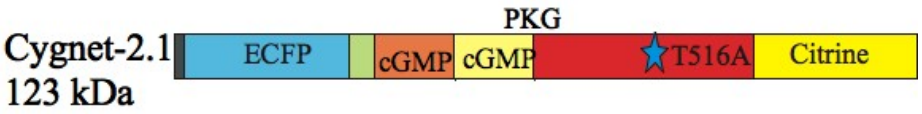

Figure II- 11 CYGNET 2.1

CYGNET 2.1 is made of PKG flanked by two YFP mutants, CFP and Citrine. Changing Threonine 516 to Alanine as shown in the figure catalytically inactivates PKG. The two cGMP binding domains of PKG are labeled cGMP in the figure (adapted from Honda, 2001)

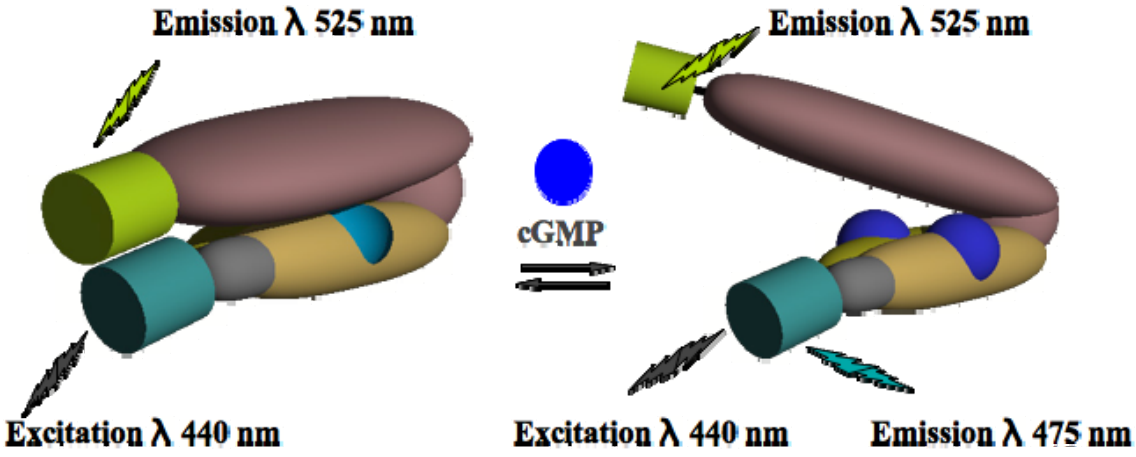

Figure II- 12 FRET with CYGNET 2.1

When cGMP is not bound to PKG, CFP and Citrine are in close proximity. Thus, when excited at 440nm, CFP emits at 475nm; this fluorescence energy is transferred to Citrine, which in turn emits at 525nm. When cGMP is bound to PKG, fluorescence energy transfer between CFP and Citrine decreases. Thus emission from Citrine decreases. Using a dual emission microscope, emissions at both 475nm and 525nm are measured to calculate the CFP/Citrine FRET ratio (adapted from Honda, 2005).

Method

Cloning of CYGNET 2.1

The CYGNET 2.1 construct was acquired from Dr. W Dostmann, UVM. Because the CFP and Citrine sequences are the same except for one amino acid change in the middle, PCR amplification of the whole CYGNET 2.1 construct was very challenging. CYGNET 2.1 was therefore cloned into the *T. gondii* expression vector in 4 steps (Figure II-13). CFP and PKG with Bgl II and Nhe I restriction sites on the 3' and 5' end respectively were generated by PCR. Digesting with Nhe 1 results in a compatible cohesive end with AvrII. A *T. gondii* expression vector was digested with Bgl II and Avr II. The CFP and PKG fragment was ligated to the expression vector. YFP already present in the vector was then converted to Citrine using site directed mutagenesis.

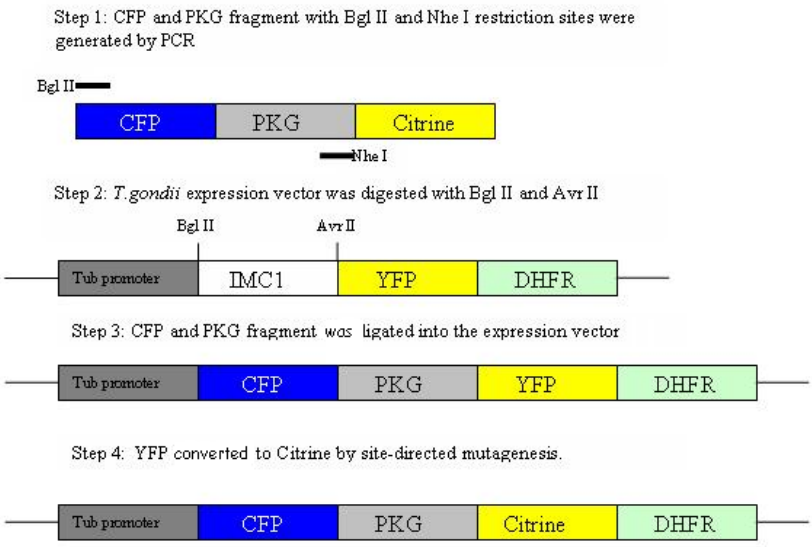

Figure II- 13 CYGNET 2.1 Cloning strategy

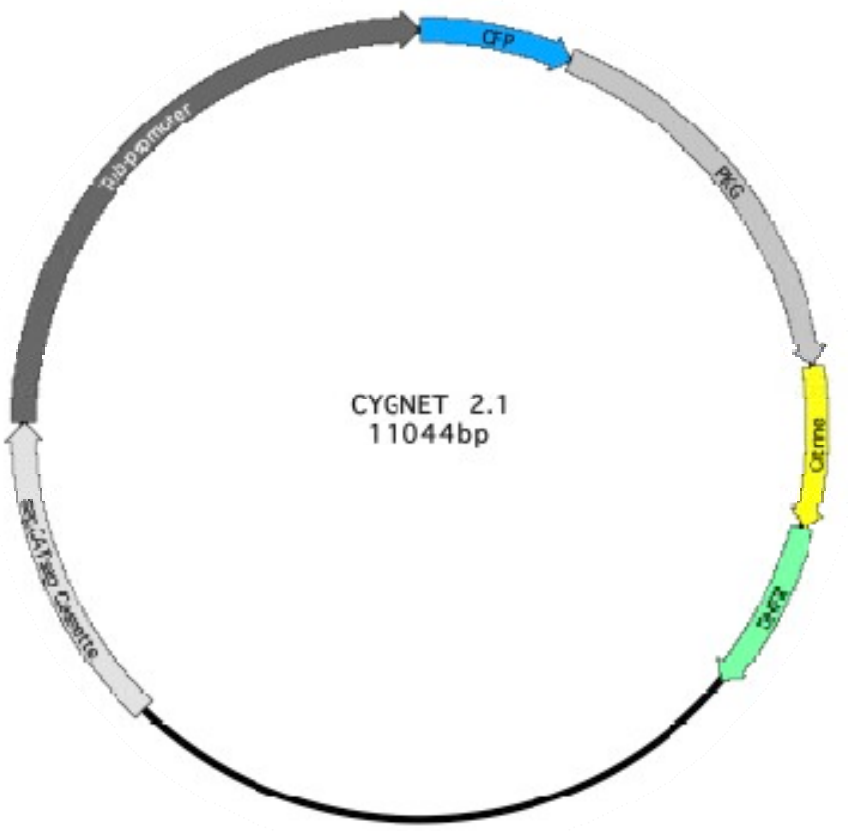

Figure II- 14 CYGNET 2.1 cloned into *T.gondii* expression vector

Transfection of T. gondii

Approximately 400 μg of the *T. gondii* expression vector CYGNET 2.1 (Figure II-14) was purified by phenol/ chloroform extraction and ethanol precipitation and resuspended in cytomix (120 mM potassium chloride, 0.15 mM calcium chloride, 10 mM potassium phosphate at pH 7.6, 25 mM Hepes-potassium hydroxide at pH 7.6, 2 mM EDTA and 5 mM magnesium chloride). Parasites were harvested and resuspended at 2 x 10^7 /ml in cytomix. 6.6 x 10^7parasites were mixed with the DNA and transfected by

electroporation. Approximately 24 hrs later, parasites were harvested and suspended at 1 x 10^7parasites/ml in 1% PBS. Parasites that emitted at 525 nm when excited at 488 nm were collected in the cell sorter. To make sure that the parasites are viable the drop drive frequency was set at 23.9 KHz and parasites collected at a rate of 800 particles/sec. Using the automatic cell deposition unit the parasites were sorted into a 96 well plate, with either 1 or 5 parasites per well. The parasites that grew up were then passaged under chloramphenicol selection.

Immunofluorescence Assay

Human foreskin fibroblasts were incubated with the CYGNET 2.1 transfected parasites overnight. Coverslips were washed and fixed in paraformaldehyde and gluteraldehyde. The primary antibodies used were rabbit anti PKG antibody (Stressgen) and rabbit anti GFP antibody (Invitrogen). Alexa 546-conjugated anti-rabbit IgG was used as the secondary antibody.

Results

I successfully generated a *T. gondii* expression plasmid containing the CYGNET 2.1 insert. Restriction digestions confirmed the presence of the insert. The efficiency of transfection of *T. gondii* with the plasmid was very low in comparison to a control plasmid in spite of optimizing the protocol. I eventually resorted to cloning out positive CYGNET 2.1 expressing parasites using the cell sorter. Positive parasites were sorted into 96 well plates. Either one or five parasites were sorted into each well. Plaques were visible only in wells containing five parasites. Once the parasites from the wells were grown out, immunofluorescence assay using antibodies against PKG and YFP were

performed after every passage. The percentage of positive parasites (Figure II-15) decreased with each passage. This suggests that expression of CYGNET 2.1 interferes with parasite viability in some way.

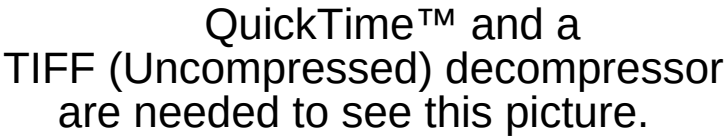

Figure II- 15 Immunofluorescence of CYGNET 2.1 expressing parasites

Immunofluorescence was performed with anti-PKG antibody and Alexa 546-conjugated anti-rabbit IgG. The phase, fluorescence and merged images are shown.

Discussion

As mentioned earlier, little is known about cyclic nucleotide signaling in *T. gondii*. Cyclic nucleotides haves been shown to play a important role during tachyzoite to bradyzoite differentiation (Kirkman, Weiss et al. 2001) and growth of *T. gondii in vitro* (Choi, Nam et al. 1990). I attempted to generate CYGNET 2.1-expressing parasite lines to measure the levels of cGMP and study its role during invasion. Unfortunately, I was unable to generate a stable parasite line expressing CYGNET 2.1. This suggests that the parasites are unable to tolerate CYGNET 2.1 expression. While I was unsuccessful in generating a stable *T. gondii* strain expressing CYGNET 2.1, parasites transiently expressing the construct could be a useful tool as well. However, it might still be a challenge to study the cGMP levels in transiently expressing parasites. The CYGNET 2.1 system has been used to study cGMP levels in vascular smooth muscle cells successfully. These cells are big and easy to see under the microscope. *T. gondii* is significantly smaller and it may be hard to visualize changes in cGMP levels. Considerable optimization will be required before we can use this tool for studies in *T. gondii*.

References

Bender, A. T. and J. A. Beavo (2006). "Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use." Pharmacol Rev **58**(3): 488-520.

Bohme, E. and G. Schultz (1974). "Separation of cyclic nucleotides by thin-layer chromatography on polyethyleneimine cellulose." Methods Enzymol **38**: 27-38.

Brier, S., D. Lemaire, et al. (2006). "Use of hydrogen/deuterium exchange mass spectrometry and mutagenesis as a tool to identify the binding region of inhibitors targeting the human mitotic kinesin Eg5." Rapid Commun Mass Spectrom **20**(3): 456-62.

Carey, K. L., N. J. Westwood, et al. (2004). "A small-molecule approach to studying invasive mechanisms of *Toxoplasma gondii*." Proc Natl Acad Sci U S A **101**(19): 7433-8.

Choi, W. Y., H. W. Nam, et al. (1990). "The effect of cyclic AMP on the growth of Toxoplasma gondii in vitro." Kisaengchunghak Chapchi **28**(2): 71-8.

Daugan, A., P. Grondin, et al. (2003). "The discovery of tadalafil: a novel and highly selective PDE5 inhibitor. 1: 5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione analogues." J Med Chem **46**(21): 4525-32.

Daugan, A., P. Grondin, et al. (2003). "The discovery of tadalafil: a novel and highly selective PDE5 inhibitor. 2: 2,3,6,7,12,12a-hexahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4-dione analogues." J Med Chem **46**(21): 4533-42.

Daugan, A. and R. Labaudiniere (2000). U.S. patent #6,143,757. U.S. patent #6,143,757. **U.S. patent #6,143,757**.

Davies, S. A. (2006). "Signalling via cGMP: lessons from Drosophila." Cell Signal **18**(4): 409-21.

Deng, W., A. Parbhu-Patel, et al. (2003). "The role of two novel regulatory sites in the activation of the cGMP-dependent protein kinase from Plasmodium falciparum." Biochem J **374**(Pt 2): 559-65.

Donald, R. G., J. Allocco, et al. (2002). "*Toxoplasma gondii* cyclic GMP-dependent kinase: chemotherapeutic targeting of an essential parasite protein kinase." Eukaryot Cell **1**(3): 317-28.

Donald, R. G., T. Zhong, et al. (2006). "Anticoccidial kinase inhibitors: identification of protein kinase targets secondary to cGMP-dependent protein kinase." Mol Biochem Parasitol **149**(1): 86-98.

Francis, S. H. and J. D. Corbin (1999). "Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action." Crit Rev Clin Lab Sci **36**(4): 275-328.

Gurnett, A. M., P. A. Liberator, et al. (2002). "Purification and molecular characterization of cGMP-dependent protein kinase from Apicomplexan parasites: A novel chemotherapeutic target." J Biol Chem **277**(18): 15913-22.

Hofmann, F., R. Feil, et al. (2006). "Function of cGMP-dependent protein kinases as revealed by gene deletion." Physiol Rev **86**(1): 1-23.

Honda, A., S. R. Adams, et al. (2001). "Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator." Proc Natl Acad Sci U S A **98**(5): 2437-42.

Honda, A., M. A. Moosmeier, et al. (2005). "Membrane-permeable cygnets: rapid cellular internalization of fluorescent cGMP-indicators." Front Biosci **10**: 1290-301.

Honda, A., C. L. Sawyer, et al. (2005). "Cygnets: in vivo characterization of novel cGMP indicators and in vivo imaging of intracellular cGMP." Methods Mol Biol **307**: 27-43.

Hotha, S., J. C. Yarrow, et al. (2003). "HR22C16: a potent small-molecule probe for the dynamics of cell division." Angew Chem Int Ed Engl **42**(21): 2379-82.

Kaupp, U. B. and R. Seifert (2002). "Cyclic nucleotide-gated ion channels." Physiol Rev **82**(3): 769-824.

Kirkman, L. A., L. M. Weiss, et al. (2001). "Cyclic nucleotide signaling in *Toxoplasma gondii* bradyzoite differentiation." Infect Immun **69**(1): 148-53.

Miyawaki, A., O. Griesbeck, et al. (1999). "Dynamic and quantitative Ca2+ measurements using improved cameleons." Proc Natl Acad Sci U S A **96**(5): 2135-40.

Miyawaki, A., J. Llopis, et al. (1997). "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin." Nature **388**(6645): 882-7.

Nakamura T, a. G. G. (1987). "A cyclic nucleotide-gated conductance in olfactory receptor cilia." Nature **325**: 442-444.

Ruth, P., W. Landgraf, et al. (1991). "The activation of expressed cGMP-dependent protein kinase isozymes I alpha and I beta is determined by the different amino-termini." Eur J Biochem **202**(3): 1339-44.

Sawyer, C. L., A. Honda, et al. (2003). "Cygnets: spatial and temporal analysis of intracellular cGMP." Proc West Pharmacol Soc **46**: 28-31.

Thompson, W. J., G. Brooker, et al. (1974). "Assay of cyclic nucleotide phosphodiesterases with radioactive substrates." Methods Enzymol **38**: 205-12.

Wall, M. E., S. H. Francis, et al. (2003). "Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase." Proc Natl Acad Sci U S A **100**(5): 2380-5.

Wiersma, H. I., S. E. Galuska, et al. (2004). "A role for coccidian cGMP-dependent protein kinase in motility and invasion." Int J Parasitol **34**(3): 369-80.

Wu, X., T. A. Haystead, et al. (1998). "Acceleration of myosin light chain dephosphorylation and relaxation of smooth muscle by telokin. Synergism with cyclic nucleotide-activated kinase." J Biol Chem **273**(18): 11362-9.

Yau, K. W. and D. A. Baylor (1989). "Cyclic GMP-activated conductance of retinal photoreceptor cells." Annu Rev Neurosci **12**: 289-327.

Zhao, J., J. Trewhella, et al. (1997). "Progressive cyclic nucleotide-induced conformational changes in the cGMP-dependent protein kinase studied by small angle X-ray scattering in solution." J Biol Chem **272**(50): 31929-36.

Zhao, Y., P. E. Brandish, et al. (1999). "A molecular basis for nitric oxide sensing by soluble guanylate cyclase." Proc Natl Acad Sci U S A **96**(26): 14753-8.

CHAPTER III

Effect of invasion inhibitors on egress

Introduction

As described in chapter 1, *T. gondii* tachyzoites can invade any nucleated cell from warm-blooded animals. They reside within the PVM and replicate. When there are approximately 128 parasites within a vacuole, the parasites lyse the host cell and egress. The process of egress is not as well characterized as invasion. However, studies on egress mutants using calcium ionophore have shown that an increase in intracellular calcium induces egress (Arrizabalaga and Boothroyd 2004; Arrizabalaga, Ruiz et al. 2004). Although it was initially thought that egress was a passive process that occurred by rupturing of the host cell membrane, it has now been shown that a moving junction, similar to that formed during invasion, forms during egress (Black and Boothroyd 2000; Alexander, Mital et al. 2005). A change in the host cell cytoplasmic potassium concentration is also thought to play a role in triggering egress. Just before egress, the PVM and host cell membrane are thought to become permeable and this results in a decrease of intracellular potassium, followed by egress of parasites (Moudy, Manning et al. 2001). To learn more about the process of egress, I tested the inhibitors of invasion identified in our high throughput screen for their effect on egress.

Method

The egress assay was performed as described (Arrizabalaga, Ruiz et al. 2004). 40mM stocks of the compounds were prepared in DMSO and diluted to appropriate concentrations in modified $HBSS_c$ (1 mM magnesium chloride, 1 mM calcium chloride, 10 mM sodium bicarbonate, and 20 mM Hepes, pH 7.2). Approximately 500 YFP expressing parasites were inoculated on a cover slip (18mm) with a confluent monolayer of human foreskin fibroblast cells. 30hrs post infection, the cover slips were washed with 1% PBS and then incubated with the compound for 15min at 23˚C. 5 μm Ionomycin (from a 1mM DMSO stock) in $HBSS_c$ with or without compound were added to the coverslip on ice. The equivalent volume of DMSO was used as a control. The coverslips were shifted to 37˚C for 5min and then were washed gently with 1% PBS. Coverslips were fixed with cold 100% methanol for 10min on ice, gently washed thrice with 1% PBS and visualized on the inverted fluorescence microscope at 20X using a YFP filter. The numbers of lysed and intact vacuoles were counted and the percentage of intact vacuoles calculated.

Results

I performed the egress assays with 8 of the invasion inhibitors. 4 out of the 8 compounds tested were non-toxic to the host cells (data not published). 2 of the compounds,144146 and 156579 do not inhibit *T. gondii* motility. The graph below represents the results from three independent assays (Figure III-1). The compounds inhibit egress to a varying extent. 267405 has no detectable effect on egress, even though it is an inhibitor of invasion and motility. Of the compounds tested, 144146 appears to inhibit egress the most.

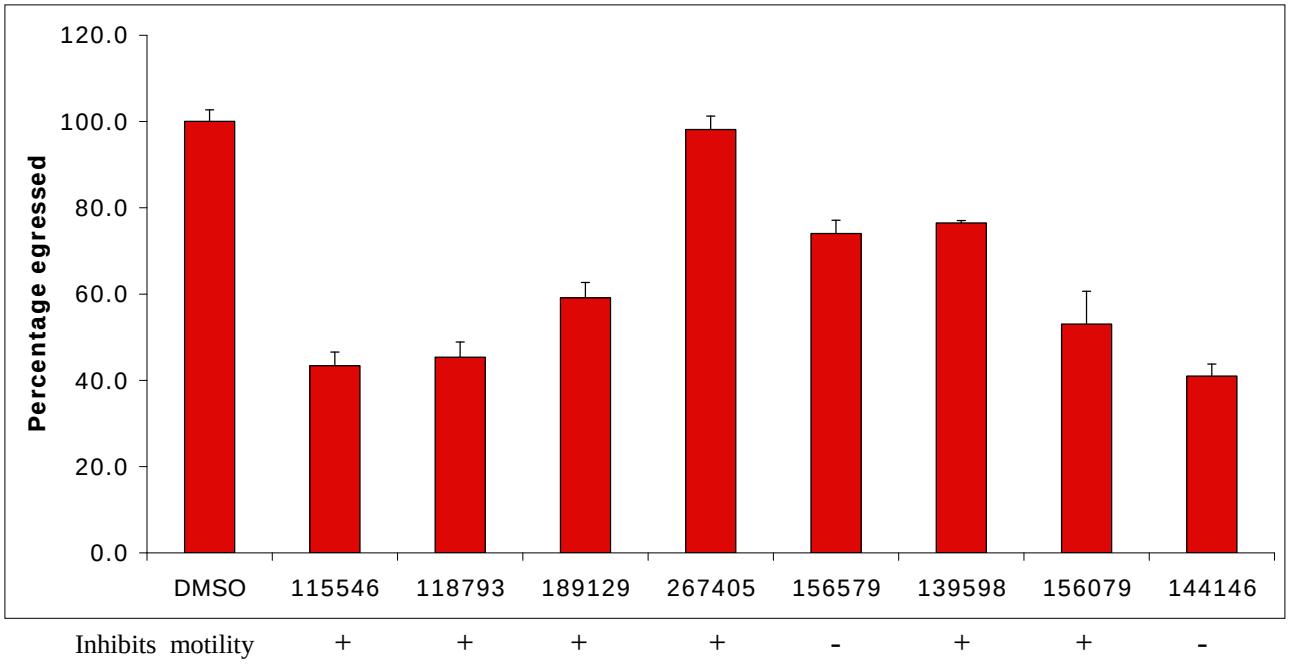

Figure III- 1 Egress assay with compounds

Values are expressed relative to the extent of egress seen after DMSO treatment (100%)

I did dose response assays with 115546 and 118793. There seems to be a direct correlation between the concentration and amount of inhibition for both compounds (Figure III-2).

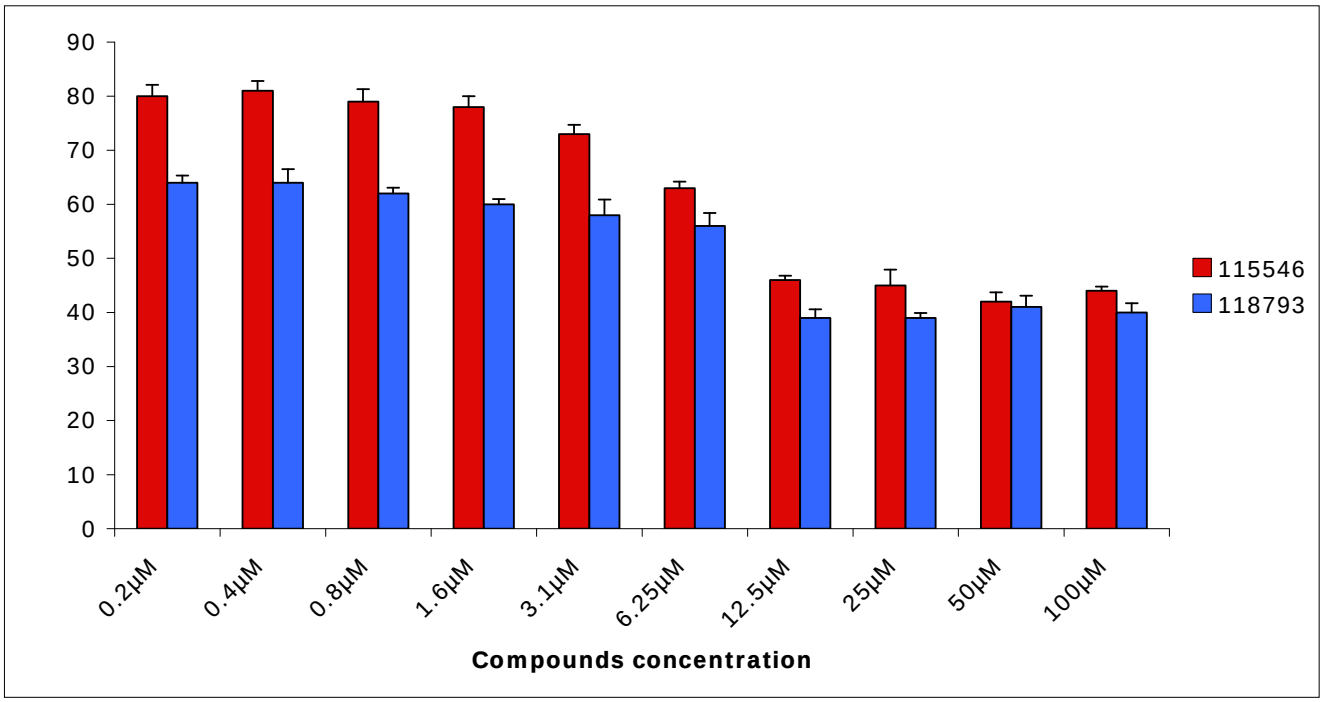

Figure III- 2 Egress assay with compounds-dose response

Values are expressed relative to the extent of egress seen after DMSO treatment (100%)

Discussion

Using the above-described assay, I have tested the effect of *T. gondii* invasion inhibitors on egress. All the compounds I tested are inhibitors of *T. gondii* invasion. However, two of the compounds, 144146 and 156579 do not inhibit motility but inhibit egress. In fact, 144146 inhibits egress to the greatest extent of any of the compounds tested. 144146 inhibits ionophore-induced conoid extension. It also appears to inhibit ionophore-induced egress. This suggests that 144146 possibly inhibits calcium signaling. It is also possible that conoid is involved in the process of egress.

The other interesting outcome of this assay was 267405 does not inhibit egress although it inhibits motility and invasion. The dogma in the field is that invasion and egress are similar processes and that both require motility (Arrizabalaga and Boothroyd 2004; Morgan, Evans et al. 2007). The result with 267405 contradicts this dogma and raises many interesting questions. It is possible that the process of egress is in fact slightly different from invasion. Maybe a different subset of proteins are used during invasion which are not essential for egress. One of these proteins may be the target of 267405. The egress assay was performed by inducing egress using a calcium ionophore. It would be of interest to test the same compounds for their effect on non-induced egress. However, non-induced egress assays require that parasites are treated with the compound at least six to seven hours before natural egress is observed (Black, Arrizabalaga et al. 2000). During the invasion assays the parasites were treated for 15 min with the compounds. In the egress assay, leaving in the compounds for six to seven hours may

cause different effect. One way to get around this would be to treat the parasites for 15min in the egress assay. However, if the effect of the compound is reversible, then by washing away the compound after a 15min treatment, I might see no phenotype. It will be interesting to test all the 24 inhibitors of invasion in the non-induced egress assay and in the induced egress assay and see if the results are the same. It will also be interesting to test the six enhancers of invasion to see if the enhancers enhance or induce egress.

References

Alexander, D. L., J. Mital, et al. (2005). "Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles." PLoS Pathog **1**(2): e17.

Arrizabalaga, G. and J. C. Boothroyd (2004). "Role of calcium during *Toxoplasma gondii* invasion and egress." Int J Parasitol **34**(3): 361-8.

Arrizabalaga, G., F. Ruiz, et al. (2004). "Ionophore-resistant mutant of *Toxoplasma gondii* reveals involvement of a sodium/hydrogen exchanger in calcium regulation." J Cell Biol **165**(5): 653-62.

Black, M. W., G. Arrizabalaga, et al. (2000). "Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress." Mol Cell Biol **20**(24): 9399-408.

Black, M. W. and J. C. Boothroyd (2000). "Lytic cycle of *Toxoplasma gondii*." Microbiol Mol Biol Rev **64**(3): 607-23.

Morgan, R. E., K. M. Evans, et al. (2007). "Targeting invasion and egress: from tools to drugs?" Curr Drug Targets **8**(1): 61-74.

Moudy, R., T. J. Manning, et al. (2001). "The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*." J Biol Chem **276**(44): 41492-501.

SUMMARY

Various biologically essential and important proteins have been identified and studied using small molecule approaches. A library of small molecules can be screened in an assay that results in a particular phenotype, followed by identification of the target of the compound. Two approaches can be taken using small molecules. The first is a non-presumptive approach. A library of structurally diverse small molecules with no known targets is screened. The second approach is to screen a library of compounds with known targets. The second approach generates hypotheses that can be tested. In our lab, a non-presumptive high throughput screen of approximately 12,000 compounds was performed to identify inhibitors of *T. gondii* invasion (Carey, Westwood et al. 2004). Another screen with 480 compounds with known targets was also performed (unpublished data).

In this thesis, I have described the work I did with invasion inhibitors identified in the above-mentioned non-presumptive high throughput screen. I tested the hypothesis that 118793 is an inhibitor of cGMP PDE and also tested the small molecules for their role in egress. It will be interesting to identify the target of compounds that inhibit invasion but not egress. It will also be interesting to identify targets of compounds that inhibit just egress and not motility. Induced egress is thought to involve active

motility of parasites (Black, Arrizabalaga et al. 2000). If we identify a small molecule that inhibits motility but not egress, then the above theory should be revisited.

During my Masters work, I have generated a tool to study cyclic nucleotide signaling in *T. gondii*. CYGNET 2.1 is sensitive to cGMP concentrations and highly specific for cGMP (Honda, Sawyer et al. 2005). Although I was unable to generate a stable parasite line expressing CYGNET 2.1, it would still be worthwhile to try measuring the cGMP levels in parasites transiently expressing CYGNET 2.1. I have also generated some preliminary data on the effect of 118793 on cGMP PDE. From these data it is hard to conclude whether 118793 has an effect on parasite PDE. However, it is clear that 118793 does not inhibit purified bovine PDE. Thus, it is possible that 118793 does not target cGMP PDE in *T. gondii*. As mentioned earlier, 118793 in structurally similar to an inhibitor of mammalian cGMP PDE. The region that interacts with the cGMP PDE enzyme is known for this inhibitor. Our collaborators have generated many different derivatives of 118793, which are modified in the region the mammalian inhibitor is thought to interact with cGMP PDE. From the SAR studies, modifying 118793 in this region does not affect 118793 inhibitory activity. This suggests that 118793 has different SAR characteristics and could have a completely different target. As mentioned in chapter 2, 118793 is identical to an inhibitor of mammalian kinesin Eg5. It might be worth revisiting the question if kinesins are a target of 118793 in *T. gondii*. Disrupting a kinesin could affect many processes in the cell, hence applying genetic strategies to study kinesins could be problematic. Thus, different methods of identifying the target of 118793 are in progress in our lab. We are trying to

radiolabel 118793 to perform drug western assays (Tanaka, Ohshima et al. 1999; Evans, Haraldsen et al. 2007). We have also generated a methotrexate-conjugated 118793 molecule for yeast three hybrid assays (Baker, Sengupta et al. 2003). 118793 could also be linked to an affinity matrix, which might prove to be a useful tool to identify its target.

Analysis of the signaling events involved in invasion is still at an early stage. Studies suggest calcium plays an important role during invasion. Intracellular calcium stores, which include acidocalcisomes, mitochondria, and the endoplasmic reticulum in *T. gondii*, trigger microneme discharge (Moreno and Zhong 1996; Carruthers and Sibley 1999). As mentioned in chapter 1, microneme proteins are important for adhesion and invasion (Carruthers, Giddings et al. 1999). It has been shown that during motility, the levels of calcium fluctuate, however during invasion the level of calcium decreases (Lovett and Sibley 2003). Whether or not 118793 inhibits cGMP PDE, it will still be worth pursuing the role of cGMP in invasion. Known inhibitors of PDE, guanylate cyclases and cGMP analogs were tested in our invasion assay. Various compounds had varying effects on invasion. These results and the study showing the importance of PKG during invasion (Wiersma, Galuska et al. 2004) suggest an important role for cGMP during invasion.

References

Baker, K., D. Sengupta, et al. (2003). "An optimized dexamethasone-methotrexate yeast 3-hybrid system for high-throughput screening of small molecule-protein interactions." Anal Biochem **315**(1): 134-7.

Black, M. W., G. Arrizabalaga, et al. (2000). "Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress." Mol Cell Biol **20**(24): 9399-408.

Carruthers, V. B., O. K. Giddings, et al. (1999). "Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells." Cell Microbiol **1**(3): 225-235.

Carruthers, V. B. and L. D. Sibley (1999). "Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*." Mol Microbiol **31**(2): 421-8.

Evans, K. M., J. D. Haraldsen, et al. (2007). "Synthesis and chemical characterisation of target identification reagents based on an inhibitor of human cell invasion by the parasite *Toxoplasma gondii*." Org Biomol Chem **5**(13): 2063-9.

Honda, A., C. L. Sawyer, et al. (2005). "Cygnets: in vivo characterization of novel cGMP indicators and in vivo imaging of intracellular cGMP." Methods Mol Biol **307**: 27-43.

Lovett, J. L. and L. D. Sibley (2003). "Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells." J Cell Sci **116**(Pt 14): 3009-16.

Moreno, S. N. and L. Zhong (1996). "Acidocalcisomes in *Toxoplasma gondii* tachyzoites." Biochem J **313**(Pt 2): 655-9.

Tanaka, H., N. Ohshima, et al. (1999). "Isolation of cDNAs encoding cellular drug-binding proteins using a novel expression cloning procedure: drug-western." Mol Pharmacol **55**(2): 356-63.

Bibliography

Achbarou, A., O. Mercereau-Puijalon, et al. (1991). "Differential targeting of dense granule proteins in the parasitophorous vacuole of *Toxoplasma gondii*." Parasitology **103 Pt 3**: 321-9.

Aikawa, M., Y. Komata, et al. (1977). "Transmission and scanning electron microscopy of host cell entry by *Toxoplasma gondii*." Am J Pathol **87**(2): 285-96.

Alexander, D. L., J. Mital, et al. (2005). "Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles." PLoS Pathog **1**(2): e17.

Arrizabalaga, G. and J. C. Boothroyd (2004). "Role of calcium during *Toxoplasma gondii* invasion and egress." Int J Parasitol **34**(3): 361-8.

Arrizabalaga, G., F. Ruiz, et al. (2004). "Ionophore-resistant mutant of *Toxoplasma gondii* reveals involvement of a sodium/hydrogen exchanger in calcium regulation." J Cell Biol **165**(5): 653-62.

Baker, K., D. Sengupta, et al. (2003). "An optimized dexamethasone-methotrexate yeast 3-hybrid system for high-throughput screening of small molecule-protein interactions." Anal Biochem **315**(1): 134-7.

Baum, J., D. Richard, et al. (2006). "A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites." J Biol Chem **281**(8): 5197-208.

Beckers, C. J., J. F. Dubremetz, et al. (1994). "The *Toxoplasma gondii* rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm." J Cell Biol **127**(4): 947-61.

Bender, A. T. and J. A. Beavo (2006). "Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use." Pharmacol Rev **58**(3): 488-520.

Black, M. W., G. Arrizabalaga, et al. (2000). "Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress." Mol Cell Biol **20**(24): 9399-408.

Black, M. W. and J. C. Boothroyd (2000). "Lytic cycle of *Toxoplasma gondii*." Microbiol Mol Biol Rev **64**(3): 607-23.

Bohme, E. and G. Schultz (1974). "Separation of cyclic nucleotides by thin-layer chromatography on polyethyleneimine cellulose." Methods Enzymol **38**: 27-38.

Brecht, S., V. B. Carruthers, et al. (2001). "The *Toxoplasma* micronemal protein MIC4 is an adhesin composed of six conserved apple domains." J Biol Chem **276**(6): 4119-27.

Brier, S., D. Lemaire, et al. (2006). "Use of hydrogen/deuterium exchange mass spectrometry and mutagenesis as a tool to identify the binding region of inhibitors targeting the human mitotic kinesin Eg5." Rapid Commun Mass Spectrom **20**(3): 456-62.

Brossier, F., T. J. Jewett, et al. (2005). "A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by *Toxoplasma*." Proc Natl Acad Sci U S A **102**(11): 4146-51.

Brydges, S. D., G. D. Sherman, et al. (2000). "Molecular characterization of TgMIC5, a proteolytically processed antigen secreted from the micronemes of *Toxoplasma gondii*." Mol Biochem Parasitol **111**(1): 51-66.

Brydges, S. D., X. W. Zhou, et al. (2006). "Targeted deletion of MIC5 enhances trimming proteolysis of *Toxoplasma* invasion proteins." Eukaryot Cell **5**(12): 2174-83.

Carey, K. L., N. J. Westwood, et al. (2004). "A small-molecule approach to studying invasive mechanisms of *Toxoplasma gondii*." Proc Natl Acad Sci U S A **101**(19): 7433-8.

Carruthers, V. B., O. K. Giddings, et al. (1999). "Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells." Cell Microbiol **1**(3): 225-235.

Carruthers, V. B., S. N. Moreno, et al. (1999). "Ethanol and acetaldehyde elevate intracellular [Ca2+] and stimulate microneme discharge in *Toxoplasma gondii*." Biochem J **342 (Pt 2)**: 379-86.

Carruthers, V. B. and L. D. Sibley (1997). "Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts." Eur J Cell Biol **73**(2): 114-23.

Carruthers, V. B. and L. D. Sibley (1999). "Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*." Mol Microbiol **31**(2): 421-8.

Cesbron-Delauw, M. F., L. Lecordier, et al. (1996). "Role of secretory dense granule organelles in the pathogenesis of toxoplasmosis." Curr Top Microbiol Immunol **219**: 59-65.

Choi, W. Y., H. W. Nam, et al. (1990). "The effect of cyclic AMP on the growth of Toxoplasma gondii in vitro." Kisaengchunghak Chapchi **28**(2): 71-8.

Daugan, A., P. Grondin, et al. (2003). "The discovery of tadalafil: a novel and highly selective PDE5 inhibitor. 1: 5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione analogues." J Med Chem **46**(21): 4525-32.

Daugan, A., P. Grondin, et al. (2003). "The discovery of tadalafil: a novel and highly selective PDE5 inhibitor. 2: 2,3,6,7,12,12a-hexahydropyrazino[1',2':1,6]pyrido[3,4-b]indole-1,4-dione analogues." J Med Chem **46**(21): 4533-42.

Daugan, A. and R. Labaudiniere (2000). U.S. patent #6,143,757. U.S. patent #6,143,757. **U.S. patent #6,143,757**.

Davies, S. A. (2006). "Signalling via cGMP: lessons from Drosophila." Cell Signal **18**(4): 409-21.

Deng, W., A. Parbhu-Patel, et al. (2003). "The role of two novel regulatory sites in the activation of the cGMP-dependent protein kinase from Plasmodium falciparum." Biochem J **374**(Pt 2): 559-65.

Donald, R. G., J. Allocco, et al. (2002). "*Toxoplasma gondii* cyclic GMP-dependent kinase: chemotherapeutic targeting of an essential parasite protein kinase." Eukaryot Cell **1**(3): 317-28.

Donald, R. G., T. Zhong, et al. (2006). "Anticoccidial kinase inhibitors: identification of protein kinase targets secondary to cGMP-dependent protein kinase." Mol Biochem Parasitol **149**(1): 86-98.

Dowse, T. and D. Soldati (2004). "Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis." Curr Opin Microbiol **7**(4): 388-96.

Dowse, T. J., J. C. Pascall, et al. (2005). "Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion." Int J Parasitol **35**(7): 747-56.

Dubremetz, J. F. (2007). "Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction." Cell Microbiol **9**(4): 841-8.

Evans, K. M., J. D. Haraldsen, et al. (2007). "Synthesis and chemical characterisation of target identification reagents based on an inhibitor of human cell invasion by the parasite *Toxoplasma gondii*." Org Biomol Chem **5**(13): 2063-9.

Fourmaux, M. N., A. Achbarou, et al. (1996). "The MIC1 microneme protein of *Toxoplasma gondii* contains a duplicated receptor-like domain and binds to host cell surface." Mol Biochem Parasitol **83**(2): 201-10.

Francis, S. H. and J. D. Corbin (1999). "Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action." Crit Rev Clin Lab Sci **36**(4): 275-328.

Gaskins, E., S. Gilk, et al. (2004). "Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*." J Cell Biol **165**(3): 383-93.

Gilbert, L. A., S. Ravindran, et al. (2007). "*Toxoplasma gondii* targets a protein phosphatase 2C to the nuclei of infected host cells." Eukaryot Cell **6**(1): 73-83.

Gurnett, A. M., P. A. Liberator, et al. (2002). "Purification and molecular characterization of cGMP-dependent protein kinase from Apicomplexan parasites: A novel chemotherapeutic target." J Biol Chem **277**(18): 15913-22.

Hoff, E. F. and V. B. Carruthers (2002). "Is *Toxoplasma egress* the first step in invasion?" Trends Parasitol **18**(6): 251-5.

Hofmann, F., R. Feil, et al. (2006). "Function of cGMP-dependent protein kinases as revealed by gene deletion." Physiol Rev **86**(1): 1-23.

Honda, A., S. R. Adams, et al. (2001). "Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator." Proc Natl Acad Sci U S A **98**(5): 2437-42.

Honda, A., M. A. Moosmeier, et al. (2005). "Membrane-permeable cygnets: rapid cellular internalization of fluorescent cGMP-indicators." Front Biosci **10**: 1290-301.

Honda, A., C. L. Sawyer, et al. (2005). "Cygnets: in vivo characterization of novel cGMP indicators and in vivo imaging of intracellular cGMP." Methods Mol Biol **307**: 27-43.

Hotha, S., J. C. Yarrow, et al. (2003). "HR22C16: a potent small-molecule probe for the dynamics of cell division." Angew Chem Int Ed Engl **42**(21): 2379-82.

Hu, K., D. S. Roos, et al. (2002). "A novel polymer of tubulin forms the conoid of *Toxoplasma gondii*." J Cell Biol **156**(6): 1039-50.

Kaupp, U. B. and R. Seifert (2002). "Cyclic nucleotide-gated ion channels." Physiol Rev **82**(3): 769-824.

Keeley, A. and D. Soldati (2004). "The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa." Trends Cell Biol **14**(10): 528-32.

Kim, K. and L. M. Weiss (2004). "*Toxoplasma gondii*: the model apicomplexan." Int J Parasitol **34**(3): 423-32.

Kirkman, L. A., L. M. Weiss, et al. (2001). "Cyclic nucleotide signaling in *Toxoplasma gondii* bradyzoite differentiation." Infect Immun **69**(1): 148-53.

Lebrun, M., A. Michelin, et al. (2005). "The rhoptry neck protein RON4 re-localizes at the moving junction during *Toxoplasma gondii* invasion." Cell Microbiol **7**(12): 1823-33.

Lecordier, L., C. Mercier, et al. (1993). "Molecular structure of a *Toxoplasma gondii* dense granule antigen (GRA 5) associated with the parasitophorous vacuole membrane." Mol Biochem Parasitol **59**(1): 143-53.

Lecordier, L., I. Moleon-Borodowsky, et al. (1995). "Characterization of a dense granule antigen of *Toxoplasma gondii* (GRA6) associated to the network of the parasitophorous vacuole." Mol Biochem Parasitol **70**(1-2): 85-94.

Leriche, M. A. and J. F. Dubremetz (1990). "Exocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion." Parasitol Res **76**(7): 559-62.

Lovett, J. L., N. Marchesini, et al. (2002). "*Toxoplasma gondii* microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores." J Biol Chem **277**(29): 25870-6.

Lovett, J. L. and L. D. Sibley (2003). "Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells." J Cell Sci **116**(Pt 14): 3009-16.

Mann, T. and C. Beckers (2001). "Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite *Toxoplasma gondii*." Mol Biochem Parasitol **115**(2): 257-68.

Mazumdar, J., H. W. E, et al. (2006). "Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in *Toxoplasma gondii*." Proc Natl Acad Sci U S A **103**(35): 13192-7.

Meissner, M., S. Brecht, et al. (2001). "Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*." Nucleic Acids Res **29**(22): E115.

Meissner, M., M. Reiss, et al. (2002). "A family of transmembrane microneme proteins of *Toxoplasma gondii* contain EGF-like domains and function as escorters." J Cell Sci **115**(Pt 3): 563-74.

Mital, J., M. Meissner, et al. (2005). "Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion." Mol Biol Cell **16**(9): 4341-9.

Miyawaki, A., O. Griesbeck, et al. (1999). "Dynamic and quantitative Ca2+ measurements using improved cameleons." Proc Natl Acad Sci U S A **96**(5): 2135-40.

Miyawaki, A., J. Llopis, et al. (1997). "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin." Nature **388**(6645): 882-7.

Mondragon, R. and E. Frixione (1996). "Ca(2+)-dependence of conoid extrusion in *Toxoplasma gondii* tachyzoites." J Eukaryot Microbiol **43**(2): 120-7.

Monteiro, V. G., E. J. de Melo, et al. (2001). "Morphological changes during conoid extrusion in Toxoplasma gondii tachyzoites treated with calcium ionophore." J Struct Biol **136**(3): 181-9.

Moreno, S. N. and L. Zhong (1996). "Acidocalcisomes in *Toxoplasma gondii* tachyzoites." Biochem J **313**(Pt 2): 655-9.

Morgan, R. E., K. M. Evans, et al. (2007). "Targeting invasion and egress: from tools to drugs?" Curr Drug Targets **8**(1): 61-74.

Morrissette, N. S., J. M. Murray, et al. (1997). "Subpellicular microtubules associate with an intramembranous particle lattice in the protozoan parasite *Toxoplasma gondii*." J Cell Sci **110**(Pt 1): 35-42.

Moudy, R., T. J. Manning, et al. (2001). "The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*." J Biol Chem **276**(44): 41492-501.

Nakamura T, a. G. G. (1987). "A cyclic nucleotide-gated conductance in olfactory receptor cilia." Nature **325**: 442-444.

Opitz, C., M. Di Cristina, et al. (2002). "Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite *Toxoplasma gondii*." EMBO J **21**(7): 1577-85.

Opitz, C. and D. Soldati (2002). "'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*." Mol Microbiol **45**(3): 597-604.

Peterson, J. R. and T. J. Mitchison (2002). "Small molecules, big impact. A history of chemical inhibitors and the cytoskeleton." Chem Biol **9**(12): 1275-85.

Pfefferkorn, E. R. and L. C. Pfefferkorn (1976). "*Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants." Exp Parasitol **39**(3): 365-76.

Roos, D. S., M. J. Crawford, et al. (1999). "Transport and trafficking: Toxoplasma as a model for Plasmodium." Novartis Found Symp **226**: 176-95; discussion 195-8.

Ruth, P., W. Landgraf, et al. (1991). "The activation of expressed cGMP-dependent protein kinase isozymes I alpha and I beta is determined by the different amino-termini." Eur J Biochem **202**(3): 1339-44.

Saeij, J. P., J. P. Boyle, et al. (2006). "Polymorphic secreted kinases are key virulence factors in toxoplasmosis." Science **314**(5806): 1780-3.

Sawyer, C. L., A. Honda, et al. (2003). "Cygnets: spatial and temporal analysis of intracellular cGMP." Proc West Pharmacol Soc **46**: 28-31.

Soldati, D., A. Lassen, et al. (1998). "Processing of *Toxoplasma* ROP1 protein in nascent rhoptries [In Process Citation]." Mol Biochem Parasitol **96**(1-2): 37-48.

Striepen, B., C. Y. He, et al. (1998). "Expression, selection, and organellar targeting of the green fluorescent protein in *Toxoplasma gondii*." Mol Biochem Parasitol **92**(2): 325-38.

Striepen, B. and D. Soldati (2007). Genetic manipulation of *Toxoplasma gondii*. *Toxoplasma gondii*: The Model Apicomplexan Perspectives and Methods. L. M. Weiss and K. Kim. London, Academic Press**:** 391-415.

Suss-Toby, E., J. Zimmerberg, et al. (1996). "*Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore." Proc Natl Acad Sci U S A **93**(16): 8413-8.

Tanaka, H., N. Ohshima, et al. (1999). "Isolation of cDNAs encoding cellular drug-binding proteins using a novel expression cloning procedure: drug-western." Mol Pharmacol **55**(2): 356-63.

Taylor, S., A. Barragan, et al. (2006). "A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*." Science **314**(5806): 1776-80.

Teo, C. F., X. W. Zhou, et al. (2007). "Cysteine protease inhibitors block Toxoplasma gondii microneme secretion and cell invasion." Antimicrob Agents Chemother **51**(2): 679-88.

Thompson, W. J., G. Brooker, et al. (1974). "Assay of cyclic nucleotide phosphodiesterases with radioactive substrates." Methods Enzymol **38**: 205-12.

Vercammen, M., T. Scorza, et al. (2000). "DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice." Infect Immun **68**(1): 38-45.

Wall, M. E., S. H. Francis, et al. (2003). "Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase." Proc Natl Acad Sci U S A **100**(5): 2380-5.

Wan, K. L., V. B. Carruthers, et al. (1997). "Molecular characterisation of an expressed sequence tag locus of *Toxoplasma gondii* encoding the micronemal protein MIC2." Mol Biochem Parasitol **84**(2): 203-14.

White, M. W., M. E. Jerome, et al. (2005). "Genetic rescue of a *Toxoplasma gondii* conditional cell cycle mutant." Mol Microbiol **55**(4): 1060-71.

Wiersma, H. I., S. E. Galuska, et al. (2004). "A role for coccidian cGMP-dependent protein kinase in motility and invasion." Int J Parasitol **34**(3): 369-80.

Wu, X., T. A. Haystead, et al. (1998). "Acceleration of myosin light chain dephosphorylation and relaxation of smooth muscle by telokin. Synergism with cyclic nucleotide-activated kinase." J Biol Chem **273**(18): 11362-9.

Yap, G. S. and A. Sher (1999). "Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function." Immunobiology **201**(2): 240-7.

Yau, K. W. and D. A. Baylor (1989). "Cyclic GMP-activated conductance of retinal photoreceptor cells." Annu Rev Neurosci **12**: 289-327.

Zhao, J., J. Trewhella, et al. (1997). "Progressive cyclic nucleotide-induced conformational changes in the cGMP-dependent protein kinase studied by small angle X-ray scattering in solution." J Biol Chem **272**(50): 31929-36.

Zhao, Y., P. E. Brandish, et al. (1999). "A molecular basis for nitric oxide sensing by soluble guanylate cyclase." Proc Natl Acad Sci U S A **96**(26): 14753-8.