TARGETING THE PROCESS OF ATTACHMENT IN GIARDIA LAMBLIA PATHOGENESIS: A NEW APPROACH IN GIARDIA DRUG DISCOVERY

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By

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DEDICATION:

This is dedicated with love to my amazing daughter for giving me the courage and conviction to pursue all that is meaningful.
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ABSTACT

*Giardia lamblia* is one of the most prevalent intestinal parasites worldwide and is a significant contributor to diarrheal diseases. Treatment of *Giardia* has proven to be difficult as there is no vaccine and the arsenal of chemotherapeutics is limited. Challenges regarding current treatments include high recurrence rates, frequency and severity of side effects, and reports of resistance and contraindications. These challenges have demonstrated a clear need to develop novel chemotherapeutics.

Parasite attachment to the epithelial lining of the small intestine is a critical step in initiating and maintaining an infection, making it an appealing drug target. Unfortunately, our ability to develop novel drugs that specifically target attachment has been hindered by our lack of knowledge regarding the mechanism, and our poor understanding of the cellular machinery involved in attachment is in turn largely due to a lack of research tools.
Here, we have devised a top-down phenotypic high-throughput inhibitor screen that targets parasite attachment. The significance of our top-down approach is two-fold; (1) identifying compounds that inhibit parasite attachment may be useful in identifying and developing novel drug candidates for the treatment of giardiasis, and (2) identifying compounds that will broaden our understanding of the process of attachment will ultimately allow us to identify a larger range of novel drug targets.

Here, we have screened 1,978 compounds from the NCI Diversity Set I, and have identified seven top lead drug candidates that significantly inhibit attachment between 5-10uM, without affecting IEC-6 host cells at a higher concentration (50uM). Currently these compounds are under review for U.S. patent rights as prospective lead compounds for chemotherapeutic development.

To further gain insights on the mechanism of attachment, we have conducted videomicroscopy of parasite morphology and behavior in response to our top lead hits. We defined 3 phenotypic categories: compounds that disrupted parasite morphology; compounds that did not affect gross parasite morphology but that caused flagellar paralysis; and most relevant, compounds that did not affect gross parasite morphology or activity, but that prevented attachment. Compounds that appear to specifically inhibit attachment are promising tools for future identification of the cellular machinery involved in *Giardia* attachment.
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CHAPTER 1
Introduction

1.1 Giardiasis

1.1.1 Clinical Manifestations

*Giardia lamblia* is a prevalent intestinal parasite and major cause of diarrheal disease throughout the world [1]. Although often asymptomatic, giardiasis can be characterized by gastrointestinal disorders, such as diarrhea, nausea and vomiting, malabsorption, weight loss, and fatigue. Symptomatic individuals typically display manifestations of the disease within two weeks of infection [2]. Importantly, transmission of the parasite, which occurs through fecal oral contact of cysts, can persist in both symptomatic and asymptomatic individuals, and while most infections are self-limiting, a portion of individuals, particularly those who are immunocompromised, suffer from chronic infections that often result in severe weight loss, and an overall impaired state of health [3]. Explanations for the usual spectrum of symptoms and severity of infection are unclear, but may result from either difference between strains or genotypes, or difference between the host immune response [4]. In addition to human infections, *Giardia* is also known to infect a wide range of animal hosts, including
domesticated cats and dogs, cattle, and other livestock, and thus raises concerns for potential zoonotic infections [5] [6].

1.1.2 Prevalence

In the U.S. *Giardia* is responsible for approximately 2.0 - 2.4 million infections annually. Although rates of infection in the U.S. are relatively low, and primarily occur in campers, or children in daycare centers [7] [8], in the developing world, where poor sanitation conditions persist and clean water is not readily available, *Giardia* infections are largely endemic [9]. Specifically, the World Health Organization (WHO) estimates that nearly 3.5 billion people worldwide live in regions without clean drinking water or proper sewage treatments, and of these individuals approximately 1 billion may be infected with *Giardia* [10] [11]. As a consequence, these individuals may contribute to the 2.2 million deaths that occur annually from diarrheal diseases [11]. Aside from poor sanitation, it is also a zoonotic disease that is frequently found in wildlife, agricultural, and pet animal reservoirs, and therefore makes it difficult to control [5]. Epidemiology studies indicate its prevalence in the developing world ranges between 20-30% [9], causing *Giardia* to be defined as a re-emerging infectious disease [5]. Given its high prevalence, it is not surprising that *Giardia* is frequently discovered in travelers with persistent diarrhea [12].
1.1.3 *Global Impact*

Although *Giardia* infections are often self-limiting, an early study indicated that approximately 15% of infected individuals develop chronic giardiasis [13]. However, more recent studies suggest that the incidences of chronic giardiasis in certain populations can range between 32% [14] and 58% [15]. Notably, those that do suffer from chronic infections, particularly children, are at risk for long-term growth retardation and delayed cognitive development [16] [17] [18] [19]. This is a particular concern in developing countries where a majority of the population is already malnourished and typically suffering from multiple parasitic infections endemic to tropical and/or sub-tropical regions. Furthermore, *Giardia* infections can significantly impact cattle or sheep, and therefore may have economic ramifications as well [20]. Given the propensity to cause widespread morbidity, *Giardia* has been classified as a NIAID Category B Priority Pathogen, and has been added to the WHO neglected disease initiative, which targets diseases that impair social-economic and developmental growth, and thus promote poverty [21].

1.1.4 *Life Cycle*

The parasite’s life cycle, which can be replicated *in vitro*, alternates between two distinct stages– the quiescent and environmentally durable cysts, and
the vegetative trophozoites [22] [23]. Until recently, *Giardia* has thought to reproduce asexually. However, current sequencing analysis revealed evidence of meiotic recombination [24], and several important genes involved in meiotic recombination [25] [26]. Although to date no sexual cycle has been identified, this new evidence calls into question the possibility of at least a partial or facultative sexual cycle [24] [27] [28] [26].

Infection is initiated through fecal oral ingestion of cysts, which are often found in contaminated drinking water or food supplies. Subsequently, excystation occurs upon entry into the lumen of the small intestine whereby the trophozoites begin to replicate. To avoid clearance by peristalsis the trophozoites attach to the epithelial cells or mucus layer lining the small intestine, resulting in colonization. Eventually, a number of trophozoites are swept downstream of the lower small intestine where encystation is triggered, resulting in the excretion of cysts [22] [23].

The cysts are substantially resistant to the environment, surviving in fresh cold water for up to 3 months, and are resistant to the standard chlorine concentrations used in most water purification systems [29]. While the necessary dose required to initiate an infection is relative low (10-100 cysts) [1] [30], a single infection can produce up to 300 million cysts per milligram of human feces [31]. With such a high prevalence (20-30%) in developing countries [9], asymptomatic shedders also pose a significant a health risk.
1.2 Giardia Pathology

1.2.1 Understanding the Mechanism of Giardia Pathology

Although often asymptomatic, Giardia infections may lead to acute or chronic diarrhea with abdominal cramping, dehydration, nausea and/or vomiting, malabsorption, weight loss, and fatigue. Despite its global prevalence, and its propensity to cause widespread morbidity, the mechanism of pathogenesis is largely unknown. Importantly, a clearer understanding of these pathophysiological processes may help identify new therapeutic targets.

To understand the parasitic mechanisms that cause clinical symptoms, researchers have relied on both a variety of animal models [32] [33] [34] [35] [36] [37] and various intestinal cell lines [38] [39] [40] [41] [42] [43] [44] [45]. Like many intestinal pathogens, Giardia is able to cause disease without penetrating the epithelial barrier, invading surrounding tissues, or entering the bloodstream [46]. While the causes of diarrhea and malabsorption are unclear, the pathology of giardiasis is generally thought to be multifactorial, with the majority of attention focused on the functional and morphological damage to the small bowel mucosa [46]. In particular, ultrastructural studies have shown a range of villus atrophy, and a flattening or disruption of the microvillus brush border of both human and animal intestinal epithelial cells [47] [48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [42]
In addition to malabsorption, intestinal hypersecretion of chloride has been observed in rodents infected with *Giardia* [62] [36], and more recently has been documented in chronic human giardiasis [63].

The morphological changes observed in *Giardia* infections can vary considerably, and there have been reports of microvilli shortening or distortion that result in brush border enzyme deficiencies, even in the absence of villous atrophy [57] [64]. Moreover, in a large classical histological examination of intestinal sections, no pathology was observed in 96% of patients with clinically established giardiasis [65]. Although there has been some correlation between the extent of small intestinal damage and the malabsorption of nutrients [66] [67], these observations have not always been consistent [49] [50] [68]. Nonetheless, numerous studies have shown *Giardia* to cause a decrease in disaccharidase activity, a hypersecretion of chloride, and a malabsorption of glucose, sodium, and water, all of which may lead or contribute to diarrhea in human giardiasis [69] [70] [57] [36] [33] [37]. Other factors that may contribute to the broad range of symptoms and severity of infection include parasite strain virulence factors, the number of ingested cysts, and the age and/or status of the host immune system, as well as the differential immune responses of infected individuals [71]. In an experimental infection by Nash *et al.* (1987) approximately 50% of infected individuals remained asymptomatic after being infected with isolates derived from
symptomatic patients [72]. This speaks to the importance of host variability in *Giardia* pathogenesis.

### 1.2.2 *Host-Parasite Interaction and Epithelial Dysfunction*

Based on early scanning electron microscopy (SEM) observations of rat and human intestinal cells infected with *Giardia*, Erlandsen and Chase (1974) suggested that mechanical blockage or functional impairment of the mucosal surface was likely responsible for the pathology associated with giardiasis [51]. Here, Erlandsen and Chase (1974) observed circular shaped lesions disperse among attached parasites throughout the microvillous border of villous epithelial cells. Theses lesions were found to correspond to the size and shape of the ventral disk. Further transmission electron microscopy (TEM) showed displaced and deformed microvilli within the disk shaped lesion, and a reduction in the thickness of the microvilli-associated enteric surface in the area of the lesion that would presumable be covered by the ventral disk. Based on these observation, Erlandsen and Chase (1974) claimed that a combination of direct injury to the microvillus border resulting from parasite attachment, and a mechanical blockage of the mucosal surface by parasite colonization, was likely responsible for the reports of decreased disaccharidase activity and vitamin B12 malabsorption, which are functionally
related to the integrity of the microvillus border [51].

In a similar experiment by Khanna et al. (1990), NMRI mice were infected with *Giardia lamblia* trophozoites, and tissue sections of the jejunum from three individual phases of infection (3-5 days pi, 9-11 days pi, and 17-21 days pi) were analyzed for mucosal damage using both scanning and transmission electron microscopy [55]. While results from the establishment phase of infection (3-5 days pi) showed little change in epithelial surface structure, a marked reduction in villous height and a severe flattening of microvilli were reported during the acute phase of infection (9-11 days pi). These morphological changes appeared to lessen during the later stage of infection (17-21 days pi), as the parasite burden declined. Similar to Erlandsen and Chase (1974), Khanna et al. (1990) concluded that the associated malabsorption in giardiasis was caused by a reduction in the absorptive intestinal surface area, which resulted from intestinal epithelial injury presumably caused by parasite attachment [51] [55]. However, it is important to note that normal mucosal appearances have been observed in symptomatic patients [48] [49] [50] [68]. This observation suggests that the intestinal injury to the mucosal surface caused by *Giardia* infections is not exclusively responsible for the associated pathogenesis.

Aside from villous atrophy and microvillous damage to the mucosal surface, *Giardia* has also been shown to reduce epithelial barrier function and
increase intestinal permeability [73] [74] [75]. Specifically, in vivo experiments with immunocompetent and athymic CD-1 mice infected with G. muris demonstrated a decreased in small intestine barrier function, which was associated with parasite colonization, but was shown to be independent of T cell function [75]. Moreover, the loss of barrier function was not observed in the stomach or large intestine, and was restored upon parasite clearance in immunocompetent mice, but not in athymic mice that failed to clear the infection. Similar results were obtained with G. lamblia sonicates in an in vitro model using a non-transformed human intestinal cell line (SCBN) [75], and more recently in a clinical study, which indicated a loss of barrier function in chronic human giardiasis [63]. These results correlate well with earlier studies demonstrating an increased macromolecular uptake in the small intestine of gerbils infected with G. lamblia. Here, macromolecular uptake increased during the peak colonization phase of infection, but diminished during the clearance phase of infection [76].

The lack of epithelial barrier function may enable luminal antigens to activate host immune-dependent pathways, and thus has significant clinical relevance [46]. Given the medical importance, the exact mechanism responsible for reducing epithelial barrier function has remained a subject of debate. Early observations showed that live parasites, Giardia sonicates, or supernatant from living Giardia cultures were able to induce rearrangement of intestinal cellular F-
actin and α-actinin. These alterations correlated with a reduced transepithelial electrical resistance in both a colonic carcinoma (Caco2) and a non-transformed human intestinal cell line (SCBN) [77]. Additional *in vitro* studies with SCBN cultured monolayers demonstrated that *Giardia* parasites disrupt tight junctional ZO-1 protein, which is also associated with increased intestinal epithelial permeability [73]. Although not conclusive, these studies suggest that the loss of barrier function during *Giardia* infections may result from focal disruption of tight junctional proteins, and/or disruption of F-actin, which is linked to tight junctional ZO-1 protein [78] [79] [75].

In addition to the disruption of intestinal tight junctional ZO-1 and F-actin, *in vitro* experiments have demonstrated the loss of epithelial barrier function in giardiasis to be associated with increased rates of enterocyte apoptosis, and dependent on caspase-3 activity, as disruption of tight junctional ZO-1, enterocyte apoptosis, and increased epithelial permeability were inhibited with pretreatment of Z-DEVD-FMK caspase-3 inhibitor [80]. Moreover, an *in vivo* correlation of epithelial dysfunction and increased rates of enterocyte apoptosis has recently been established using TUNEL labeling of intestinal biopsies in patients with chronic giardiasis [63]. These results support earlier microarray data that examined the host-parasite interaction of human Caco2 cells and *G. lamblia* parasites. The microarray analysis indicated a significant up-regulation of genes involved in
apoptotic cascade events, and the production of reactive oxygen species [81].

1.2.3 The role of T cells in Intestinal Epithelial Damage

While it appears that disaccharidase deficiency, hypersecretion of electrolytes, and a malabsorption of glucose, sodium, and water play a role in human giardiasis, the exact mechanism or cascade of events that cause diarrhea are unclear [46]. As discussed above, ultrastructural studies have shown Giardia to cause damage to the intestinal mucosal surface, including the microvillus brush border [51] [55] [37]. However, more recent insights suggest damage to the mucosal surface and microvillus brush border is not a direct result of parasite interaction, but instead is mediated by host immune factors [82] [83] [84]. In particular, T lymphocytes appear to play an intricate role and have been implicated in the manifestation of villous atrophy for other intestinal disorders [85] [86] [87] [84]. While it is not clear what initiates the host immune response, the most current studies suggest that parasite products may act to break the epithelial barrier leading to T cell activation. As a result, the brush border may retract, causing disaccharidase deficiencies and malabsorption [37] [12] [46].

Studies by Stevens et al. (1978) initially demonstrated the importance of thymus-dependent lymphocytes in the clearance of primary infections with G.
**G. muris.** While immunocompetent BALB/c mice were able to clear parasites and developed immunity to secondary infections, congenitally hypothyemic (nude) mice failed to clear infections and were susceptible to secondary and chronic infections [88] [71] [83] [84]. Moreover, additional experiments by Roberts-Thompson *et al.* (1978) showed reconstitution of nude mice with lymphocytes from thymus-intact mice previously exposed to *Giardia*, resulted in a rapid reduction of cysts production and parasite burden. However, notably, while the reconstitution of T cells in nude mice eliminated *Giardia* infections, it also led to a marked increase in villous atrophy [89] [71] [84].

In a similar experiment, immunocompetent CD-1 mice infected with *G. muris* showed a diffused loss of the microvillous surface area, which was not observed in infected nude mice. The microvillous alterations observed in the immunocompetent mice correlated with a decreased in maltose and sucrose activity, which typically leads to intestinal malabsorption of electrolytes, nutrients, and water [82] [84]. Other studies have shown T cell induction of villous atrophy and a reduction of microvillous surface area in the absence of *Giardia* [90] [86]. These studies suggest that T cells have a functional role in both the elimination of *Giardia* infections, and the intestinal injury associated with *Giardia* pathogenesis [84].
To further examine the individual impact of CD4$^+$ and CD8$^+$ T cells on mucosal injury, Scott et al. (2004) isolated and independently transferred either CD4$^+$ or CD8$^+$ cells from the lymph nodes of Giardia infected CD-1 mice to naive immunocompetent mice. Transmission electron microscopy showed mice that received CD8$^+$ enrichments, but not CD4$^+$, had diffused shortening of microvilli, loss of brush border surface area, and increases crypt/villous ratios. Thus, the activation of CD8$^+$ T cells appears to be primarily responsible for the morphological alteration of the mucosal surface during Giardia infections [84] [91].

Together, the above findings clearly demonstrated that T cells play a functional role in both the clearance of parasites and in the observed mucosal injury observed during Giardia infections [88] [89] [82] [84]. Moreover, while CD4$^+$ T cell activation is primarily responsible for parasite clearance, epithelial mucosal injury appears to be mediated by the activation of CD8$^+$ T cells [91]. Importantly, while this indicates that the intestinal dysfunction associated with giardiasis is not exclusively a function of parasite attachment or parasite virulence factors [84] [46], it is necessary to note that parasite attachment is required and directly responsible for initiating and establishing an infection. Additionally, due to the close proximity of parasites to the epithelial surface during infections, CD8$^+$ activation is likely enhanced by parasite attachment, and thus may indirectly play a significant role in
the pathogenesis of *Giardia* infections.

### 1.3 Therapeutics for Giardiasis

#### 1.3.1 The Demand for Novel Drugs

Historically, much of drug discovery in *Giardia* has occurred by chance. Particularly, with the discovery of 2-nitroimidazole against *Trichomonas* [92], numerous derivatives including, metronidazole [93] [94] [95], benzoylmetronidazole [96], nimorazole [97], tinidazole [98], ornidazole [99], and secnidazole [100] have been tested for their activity against *Giardia*. Similarly, quinicrine, one of the first and for many years only treatment for giardiasis, was originally used to treat malaria [101]. Prior to quinacrine, harsh treatments included, mercury, tetrachloride, arsenicals, and bismuth [102]. The lack of *Giardia* targeted drug design raises the question of whether current drug therapies are the most efficient and/or effective means for treating giardiasis. Undesirable and potentially harmful side effects that frequently lead to noncompliance, reports of resistance, and high rates of reoccurrence, suggest otherwise [103] [104] [101] [105] [106].

In the U.S. the most common treatment for giardiasis is metronidazole,
which is typically less than 90% effective with reoccurrence rates as high as 90% [107] [108]. Moreover, drug resistance of up to 20% has been observed [109] [110] and examples of multidrug resistance have been reported [103] [106] [105]. Alternative drug treatments include, nitazoxanide, tinidazole, furazolidone, quinacrine, and the benzimidazole derivatives, albendazole and mebendazole. However, they all have reports of treatment failure, adverse drug reactions, and examples of resistance in the laboratory [111] [104] [112].

Additional concerns regarding current treatments include the potential for serious health risks. While there is no evidence in human studies [113], both metronidazole and tinidazole have demonstrated to be mutagenic in bacteria [114] [115], and both metronidazole and furazolidone have shown to be carcinogenic in animals [116] [101]. Additionally, quinacrine, which is able to cross the placenta, has been contraindicated for use during pregnancy due to possible links with spina bifida and renal agenesis [112]. Likewise, concerns regarding potential genotoxic and cytotoxic effects [117] have generated caution for the use of tinidazole in pregnant women, especially during the first trimester [118]. Although widely used, both mebendazole and albendazole have demonstrated to be teratogenic in animals, and thus have evoked concerns regarding its use during pregnancy [101]. Nonetheless, numerous studies failed to demonstrate a teratogenic and/or fetotoxic effect in humans [119] [120] [121] [122], and therefore they continue to be a
mainstay treatment for various parasitic infections, including *Giardia* [101]. Given the reduced effectiveness and potential health risk of these drugs, coupled with adverse reactions, and increasing drug resistance, the need to develop novel drug therapies specifically targeted against *Giardia* is apparent.

### 1.3.2 Summary of Current Treatments

**Metronidazole**

Metronidizole has been the gold standard for treatment of giardiasis in the U.S. for nearly 50 years. It belongs to a class of drugs known as the nitroimidazoles, but is commercially known as Flagyl. The drug’s mode of action is dependent on the parasite’s anaerobic metabolism, and thus becomes active when *Giardia* ferredoxins donate electrons, thereby reducing a nitro group on the drug. Once activated, metronidizole is preferentially transported into the parasitic cells, where it accumulates and interacts with cellular components, resulting in cell death [123] [112] [101]. In particular, reduction of metronidizole produces toxic radicals that interfere with parasite DNA replication, and since metronidizole can act as an alternative electron acceptor, it may also inhibit oxygen consumption by the parasite [124] [102] [112]. Side effects associated with metronidizole appear to be dose-related, and include a metallic taste, nausea, headache, vertigo, leukopenia,
irritability, and insomnia. Additional concerns include CNS toxicity, a rare but more severe side effect associated with higher doses [112] [118].

*Tinidazole*

Tinidazole, a common alternative to metronidazol, is also a member of the nitroimidazoles. Although widely used throughout Europe and the developing world as a treatment for a variety of amoebic and parasitic infections, the FDA only recently approved it in 2004 for the treatment of trichomoniasis, giardiasis, and amebiasis [125] [126]. Tinidazole has been shown to have an *in vitro* advantage over metronidizole [127] [128] [101]. Likewise, reports have indicated symptoms such as diarrhea may lessen sooner in comparison to metronidazole treatments [129], and overall cure rates appear to be higher [127] [129], or at least equivalent [130]. Tinidazole also has the advantage of being better tolerated than metronidazole. Nonetheless, side affects including a bitter taste, nausea, and skin rashes have been reported [112] [101].

*Quinacrine*

Quinacrine was the first drug used to effectively treat giardiasis. While the mechanism of action is not completely understood, the drug intercalates with *Giardia* DNA, and is thought to inhibit DNA synthesis [112]. Quinacrine has a tested efficacy rate > 90%, but due to undesirable side affects that result in
noncompliance, it’s actual efficacy rate is often reduced [102]. Potential side affects include, a bitter taste, headaches, nausea, vomiting, discoloration of skin and urine, blue or black pigmentation of the nails, sclerae, urticaria, and exfoliative dermatitis [101]. With the later discovery of metronidazole, the use of quinacrine declined, and eventually in 1992, U.S. production of the drug was discontinued [112].

Furazolidone

Furazolidone has an efficacy rate of ~80%, and thus is considerably less effective than metronidazole, tinidazole, and quinacrine [101]. However, it is available in a liquid form, making it advantageous in the treatment of infants and young children. While the mode of action is not clear, parasite death correlates with the toxicity of reduced products, which can damage DNA, as well as other cellular components [112]. Similar to other anti-giardia treatments, side affects include gastrointestinal symptoms, such as diarrhea, nausea, and vomiting [101].

Albendazole and Mebendazole

The benzimidazole derivatives, albendazole and mebendazole, are primarily thought to bind to *Giardia* β-tubulin, inhibiting microtubule polymerization and impairing glucose uptake [131] [132] [133]. Although the drugs are relatively well
tolerated, their efficacy rates are highly variable. While in vitro studies have showed promising results, demonstrating that both albendazole and mebendazole inhibited *Giardia* attachment at lower concentrations [134], and were 30 to 50 fold more active than metronidazole [131], in vivo clinical studies have shown less consistent results. Specifically, numerous studies [112] have shown efficacy rates that range from 0 - 95% for mebendazole [135] [136] [137] [138], and 24 - 100% for albendazole [139] [140] [141] [142] [143] [144] [145].

### 1.4 Biology of a Primitive Eukaryote

#### 1.4.1 Cell Structure of *Giardia lamblia*

Although the medical relevance of *Giardia* infections is apparent, its proposed early divergence on the base of the eukaryotic branch in the archaenzoan family, Hexamitidae makes *Giardia* an interesting organism to study from an evolutionary perspective [146] [147] [148] [149]. In addition to its proposed early divergence, its parasitic nature has potentially played an evolutionary role in the unusual structural features and molecular strategies it uses to carry out conventional eukaryotic cellular processes. Importantly, these unusual features, both cellular and molecular, serve as useful drug and vaccine targets that could potentially be exploited.
*Giardia* is typically described as tear-shaped with four pairs of symmetric flagella distributed bilaterally. The flagella are directed toward the caudal end to promote an anterior direction of motility. Attachment is permitted by the ventral disc, a complex structure consisting of microtubule and fibrous structures, including actin [22] [150] [151]. Other unusual features of *Giardia* include a lack of mitochondria, peroxisomes, nucleoli, and a highly plastic genome, which is supported by evidence of a vast array of VSP genes [152], frequent rearrangements of chromosomes containing rRNA genes [153] [154], and broad *in vivo* karyotype variation among isolated strains [155].

1.4.2 *The Two Nuclei*

Although it is not uncommon to observe other bi-nucleated protozoans, such as *Paramecium* and *Tetrahymena*, the genomic structure of *Giardia’s* two nuclei is quite unique. Unlike *Giardia*, bi-nucleated Ciliates contain two nuclei that are morphologically distinct. While the micronuclei, which although contains the entire genome, is only involved in sexual reproduction and is not transcribed, the macronuclei contains multiple copies of genes, which are transcribed but are not involved with sexual reproduction. In contrast, electron micrograph images of *Giardia* trophozoites indicate that the two nuclei, which reside in the anterior
portion of the cell and are symmetric about the longitudinal axis, appear morphologically identical [150] [22]. Although examples of *Giardia* with a single nucleus have been observed, currently no cell line encompassing a single nucleus has been reported, indirectly suggesting that both nuclei are required. Nonetheless, to date the functional role or redundancy of the two nuclei remains unclear. Early studies by Wieshahn, et al. (1984) involving autoradiographic analysis and density comparison of nuclear replication with tritiated thymidine show the two nuclei replicate at approximately the same time [156]. Additionally, propidium iodide [157] and DAPI nuclear staining [158] has shown the two nuclei to be equivalent in content, and metabolic labeling of RNA has shown both nuclei to be transcriptionally active [158]. Although the DNA content of each nucleus is not fully understood, *in situ* hybridization with RNA probes has shown the nuclei to be equivalent in terms of rDNA sequences [158] and fluorescent *in situ* hybridization (FISH) has shown that genes from each of the five chromosomes are present in both nuclei [159]. Therefore, in accordance with studies examining the transcriptional activity and DNA content of each nucleus, most investigators agree that each nucleus contains at least one complete copy of the genome and that both nuclei are transcriptionally active.
1.5 Genetic Variation in *Giardia*

1.5.1 Identification of *Giardia* Strains and Genotypes

Efforts to identify specific *Giardia* genotypes have been implemented by zymodeme analysis [160][161][162][163][164], restriction fragment length polymorphism analysis (RFLP) [165][166][167][168], and sequence analysis of various genes, both within a population, and among isolated populations [169][170][171][172][173][174]. Studies involving zymodeme analysis of multiple loci has putatively assigned all human and a number of mammalian isolates into two major assemblages, A and B, which includes four different subtypes, 1 and 2 belonging to assemblage A, and 3 and 4 belonging to assemblage B [172][161][162]. Electrophoretic analysis at 27 enzyme loci showed that assemblage A and B isolates had genetic differences that were relative in magnitude to those found between *G. intestinalis* and *G. muris* [162]. Zymodeme and RFLP analysis of multiple laboratory isolates by Homan et al. (1992) also identified two major groups, Polish and Belgium, that based on partial sequence analysis of the glutamate dehydrogenase gene appear to correlate to assemblages A and B, respectively [164][171]. Alternatively, three distinct genotypes, termed Nash groups 1, 2, and 3, were identified on the basis of different excretory-secretory products and surface antigens, expression of particular variant-specific
surface proteins (VSPs), and expression of a group 3 specific gene [175] [176] [177]. Although classification among *Giardia* genotypes remains debatable, sequence comparison of the triose phosphate isomerase gene indicates that Nash groups 1 and 2 correlate to assemblage A, while Nash group 3 correlates to assemblage B [170]. As further evidence to support the correspondence of these groups, a higher degree of similarity has been observed in Nash group 1 and 2 isolates (assemblage A), whereas Nash group 3 isolates (assemblage B) are highly divergent [176] [169]. Additional assemblages have also been identified in dogs (C and D) [178], livestock (E) [179], cats (F) and rats (G) [180].

1.5.2 *Heterozygosity and Implications of a Sexual Cycle*

With the completion of the *Giardia* genome project [181], a great deal of interest has been directed towards investigating levels of heterozygosity, both between the two nuclei of an individual cell, and among isolated populations. Interestingly, initial findings from the genome project have implicated extremely low levels of heterozygosity [181]. Given the conventional status as an ancient asexual organism, and the current speculations regarding a possible sexual cycle, this feature has raised many questions. Typically, low levels of heterozygosity are observed in sexually reproducing species as a result of meiotic recombination.
However, in asexual populations an accumulation of mutations over a large evolutionary distance is predicted to create divergent clonal populations, particularly in such an early diverging eukaryote. This has been demonstrated in the ancient asexual lineage of bdelloid rotifers [182]. Interestingly, as a result of the proposed equational division in which each daughter cell receives a copy of both the left and the right nuclei [159], one would predict an accumulation of mutations, particularly at the DNA level, leading to higher levels of heterozygosity between the two nuclei within a single cell. However, although sequence variation between the two nuclei has not been directly investigated, recent sequencing analysis that included both inter-isolate and intra-isolate comparisons, indirectly suggests that the genetic contents of the two nuclei are virtually identical at the sequence level [174].

1.6 The Cytoskeleton

1.6.1 Structure and Function of the Eukaryotic Cytoskeleton

In general, eukaryotic cells are defined by their highly developed cytoskeleton structures, which are primarily comprised of proteins that assemble to form three types of filaments including, intermediate filaments, microtubules, and microfilaments (reviewed in [183] [184] [185] [186]). These filaments, in
conjunction with their associated proteins, are known to play a critical role in cell motility, including both cellular division and locomotion, and are fundamental to the cell’s spatial organization and structure. Each filament is comprised of smaller units of proteins that can quickly assemble or disassemble, allowing the smaller units of protein to diffuse into the cytoplasm. This function enables cells to undergo rapid structural reorganization. Specifically, intermediate filaments are made up of a large and heterogeneous group of proteins that form string-like fibers, whereas microtubules consist of \( \alpha \)- and \( \beta \)- tubulin heterodimers that form long rigid cylinders, and microfilaments assemble in a head to tail formation with free actin monomers (G-actin) to produce a two-stranded helical filament (F-actin). A fourth category of proteins that are essential to the cytoskeleton includes motor proteins, which act to move organelles or other proteins along the different filaments.

1.6.2 The Structure and Function of the Giardia Cytoskeleton

The Flagella

While the *Giardia* cytoskeleton remains largely uncharacterized, several novel structures and proteins have been identified from early microscopy studies [187] [188] [189] [190] [191] [192] and classical biochemical assays [193] [194] [22] [150] [151] and [195]. Typically, *Giardia* trophozoites are described as tear-
shaped and are approximately 12-15 um long and 5-9 um wide with four pairs of symmetric flagella (anterior, posterior-lateral, ventral, and caudal) that are distributed bilaterally. Unlike most flagellated organisms, *Giardia* flagella are anchored deeply within the cell (reviewed in [196]). An electron dense matter, known as the dense rods, border the anterior and posterior-lateral flagella on the posterior side of the parasite [197], and short arrays of microtubules, known as the funis, are associated with the caudal flagella on the dorsal and ventral sides [198]. Although the flagella clearly play a role in motility, their role in attachment is not known.

*The Median Body*

Among the distinguishing features of *Giardia* include, the median body, which is described as a disorganized array of microtubules, located just posterior to the ventral disk and dorsal to the caudal flagella [187]. Although the median body is present in all species, its function is unknown [151] [195].

*The Ventral Disk*

Undoubtedly, the most prominent and distinct feature of *Giardia* is a large disk located on the anterior portion of the parasite’s ventral surface, just above the plasma membrane. Interestingly, the ventral disk is completely unique to *Giardia*
and is not present in other Diplomonads. The disk consists of spiral arrays of microtubules, which create a dome shaped structure [187] [188] [189] and [199] that confers increased concavity while cells are attached [60]. Numerous projections extending from the wall of each microtubule have been observed, as well as ribbon structures, which are responsible for the striated appearance [191]. The presence of microtubules and ribbon structures in the center of the disk has not been observed, and therefore, has been termed the “bare zone”. The microtubules and ribbon structures also disappear at the periphery of the disk where a network of fibers, known as the lateral crest, appears [188] [189]. The cell body continues beyond the anterior edge of the disk producing a flexible overhang termed the ventro-lateral flange, which together with the lateral crest have been observed interacting with epithelial cells, suggesting a role during attachment [187] [60].

1.6.3 The Role of the Cytoskeleton in Pathogenesis

This early divergence has enabled Giardia to independently evolve over a considerably long evolutionary distance. Given its early divergence and the unique selective pressures associated with having a parasitic nature, it is not surprising that Giardia has acquired an elaborate and unique cytoskeleton. Importantly, while these unique features are essential to Giardia, they also represent important characteristic differences from other eukaryotes, in particular that of human and
other mammalian cells. These marked differences are critical in identifying potential drugs and/or vaccines targeted against _Giardia_. Moreover, with the completion of the _Giardia_ genome project [181], we now have the tools necessary to perform in depth computational and comparative analysis of _Giardia_ cytoskeleton proteins and their respective gene structure. Accessibility to these tools will aid in the identification of novel drug targets, based on both their role in pathogenesis, and/or their sequence and structural divergence.

Notably, there is a strong link between the cytoskeleton and the pathogenesis of _Giardia_. In particular, in order to prevent clearance by peristalsis, and thus establish and maintain an infection, _Giardia_ must be able to attach to the epithelial cells lining the small intestine. Although the mechanism of attachment is unclear, the ventral disk, which is primarily composed of microtubules, and microfilaments, is believed to play an essential role in facilitating attachment. Furthermore, the cytoskeleton is involved with many common, yet essential cellular processes such as, motility, cell morphology, cell division, and internal cellular organization and trafficking. Thus, the importance of the cytoskeleton in pathogenesis, as well as basic cellular processes make it an appealing drug target.
1.7 Proposed Mechanisms of Attachment

1.7.1 Significance and Requirements of Attachment

To avoid clearance by peristalsis, and thus maintain an infection, *Giardia* must attach to epithelial cells lining the small intestine of its host. Moreover, due to the continuous shedding of intestinal cells *Giardia* must be capable of detaching, swimming, and reattaching to more stable substrates within the small intestine [151] [195]. Although these behaviors have been observed *in vitro* on both biological (various mammalian cell lines) and inert (plastic and glass) substrates [200], the mechanism of attachment and detachment remains unclear. On a fundamental level, experiments performed by Gillin and Reiner (1982) showed that attachment is dependent on temperature, pH, and ionic strength, and that attachment is most optimal at physiological conditions [201]. Additionally, in order to attach, *Giardia* must be biologically active, as glutaraldehyde fixation [40] [60], inhibition of glycoysis by iodoacetate [202], and temperatures below 12°C [201] have all shown to inhibit attachment. While the exact mechanism of attachment remains elusive, several theories have emerged, none of which are mutually exclusive, and all of which have supporting, but not conclusive evidence (reviewed in [151] [195]).
1.7.2 *The Hydrodynamic Model of Attachment*

Early microscopy observations of Giardia in relation to host epithelial cells by [188] [189] and [203] led to a hydrodynamic model of attachment, in which [190] used a viscous-flow equation to suggest that negative pressure generated from a fluid flow led beneath the cell by flagellar activity produces a suction force enabling Giardia to remain attached. However, the model does not account for detachment, as observations demonstrate the ventral flagella beat continuously regardless of cell activity (reviewed in [151] [195]). Moreover, Holberton’s calculations were based on an incorrect morphology model that assumed the ventral flagella emerged from the center of the disk, whereas later studies by [191] showed that the ventral flagella emerge from the cell body in the ventral groove, dorsal to the ventral disk.

1.7.3 *Adhesive-Mediated Attachment*

To examine the adhesive role of the ventro-lateral flange in attachment Erlandsen et al. (2004) constructed microfabricated substrates with pillars that sterically inhibited the formation of the hypothesized negative pressure under the ventral disk. Parasites were primarily observed to attach to the flat surfaces of the substrate, while a dramatic reduction in parasites attachment was observed in the
pillar regions (16% of controls). Erlandsen argued that the principal mechanism of attachment is mediated via the ventral disk, but that membrane adhesion of the ventro-lateral flange may have a secondary role in attachment [204].

1.7.4 *Lectin-Mediated Attachment*

Given that *Giardia* cells must be in correct orientation for mechanical or hydrodynamic forces to act in attachment, numerous groups have, in part, attributed attachment to surface mannose-binding lectins, which bind to receptors on host epithelial cells. This in theory would provide a primary “braking mechanism” which would act over the entire surface of the parasite allowing them to stick before adapting a more suitable orientation for attachment [205]. Interestingly, Inge et al. (1988) showed that *Giardia* preferentially attached to intestinal epithelial cells of the small intestine relative to colon cells, which is unexplained solely by a mechanical mechanism, and thus, supports a role for lectin interaction not only in correct orientation during attachment, but also in cell specificity [38]. Although many studies have focused on the role of lectins in attachment, results have largely been inconclusive [42] [44]. Nonetheless, while it seems that lectin interactions may play a role in attachment, it is also important to note that *Giardia* is capable of attaching to glass and plastic substrates [200] with high efficiency, indicating that
cytoskeleton mechanisms are adequate for attachment.

1.7.5 Cytoskeleton-Mediated Attachment

As an alternative mode of attachment other investigators have directed their focus towards the intricate role of the ventral disk. An early theory put forth by Mueller et al. (1974) suggested that Giardia attachment was mediated by tightening the spiral array of microtubules that make up the ventral disk, in what was describe as a clutching like mechanism [199]. However, early microscopy studies by Holberton (1973a,b, 1974) revealed that the dorsal ribbons of the disk are linked by filaments, which would break if the disk were allowed loosen and tighten in a clutching like fashion [188] [189] [190] (reviewed in [151, 195].

Numerous micrograph images of parasites attached to epithelial cells have shown a prominent contractile or grasping motion at the edges of the disk, which appears to act like a hook displacing the microvillus during attachment [187] [188] [206] [51] [207] [40] [60] and [204]. Additionally, distinct marks or “footprints” that correspond in size and shape to the lateral crest have frequently been observed on host epithelial cells after parasites have detached, implying that either contractile and/or suction forces generated by the ventral disk may play a dominate role in attachment [206] [51] [42] [60] [208] [60]. This is supported by the localization of
actin to the periphery of the cell, lateral crest, and ventral disk, in addition to a network localized under the dorsal plasma membrane (Khoury and Elmendorf, unpublished). To further substantiate this, multiple drug studies using microfilament disrupting drugs have shown to inhibit parasite attachment [202] [209] (Khoury and Elmendorf, unpublished). Furthermore, a more recent study indicated that *Giardia* detachment forces were not significantly different for parasites attached to a positively charged hydrophobic substrate or inert surfaces, when compared to parasites attached to untreated glass [210]. Together, these findings are consistent with a suction based model of attachment, and argue that although molecular binding and/or adhesion may have a prominent role in attachment, it is more likely to serve as a secondary mechanism that aids in the recognition of more suitable cell substrates. Thus, it is reasonable to conclude that a suction model is primarily responsible for facilitating the parasite attachment required for sustaining an infection [195].
1.8 *Giardia* Attachment: Summary of Previous Drug Studies

1.8.1 *Targeting the Process of Attachment*

While the exact mechanism of attachment remains elusive, it has become increasingly clear that the cytoskeleton, and primarily the ventral disk, has a prominent role in attachment, and thus is essential in initiating and maintaining infections. Since maintenance of the cytoskeleton, including the ventral disk, requires the assembly and disassembly of filaments, in particular microtubules and actin microfilaments, many drug studies have focused on the disruption of these filaments to identify their role in attachment [202] [211] [38] [40] [42] [44] [60]. However, most of these studies have been complicated by the use of various cell lines as a substrate for attachment, including rat intestinal epithelial cells (enterocytes) [38], rat small intestine cells (IEC-6) [40], human cancer colonic epithelial cells (Caco-2) [42] [44], and human embryonic jejunum and ileum cells (Int-407) [60]. Specifically, in studies [42] [44] [60] where drug treatments were applied during a co-incubation with substrate cells, it is difficult to delineate whether the observed drug effects were a result of drug interaction with the parasites, or the substrate cells, which may indirectly alter parasite attachment. Additionally, drug effects can be rapidly reversible, making it difficult to observe an effect when parasites were pretreated prior to incubation with substrate cells [38].
Unfortunately, while earlier studies examining *Giardia* attachment to inert substrates in the presence of drugs, are considerably more relevant, poor experimental design has also lead to contradictory and unreliable results.

1.8.2 *Microtubules V. Microfilaments*

Feely and Erlandsen (1982) [202] examined *Giardia* attachment in the presence of the microtubule disruptor colchicine and the microfilament disruptor cytochalasin-B. Here, *Giardia* trophozoites were isolated from the small intestines of rats and allowed to attach during a 20 minute incubation in Petri dishes at 37° C in HBSS (pH 7.2). The parasites were gently washed twice with HBSS and then each dish received 2ml of either colchicine dissolved in HBSS (1000uM, 10uM and 0.1uM), or cytochalasin-B, dissolved in DMSO, and then diluted in HBSS (10mg/ml). Controls included treatments of 1% DMSO and HBSS in the absence of drugs. To determine an initial cell count, parasite counts of five random fields were done using a reticle eye piece, and the average number of attached cells per plate was determined by multiplying the average number of trophozoites per field by the ratio of the plate area to the field area. The average number of cells for all plates was use to express 100% attachment at time point 0. After the initial cell
count was determined, cells were incubated at 37°C for 60 minutes and washed with HBSS at 10-minute intervals. Cell counts were determined after each wash and statistical significance was determined by analysis of variance. Their results showed that cytochalasin-B significantly reduced *Giardia* attachment by ~26% after 30 minutes and ~35 % after 50 minutes. However, under similar conditions, they reported that colchicine had no effect on attachment, although data were not shown.

While Feely and Erlandsen [202] did show a significant decrease in attachment when cells were treated with the microfilament disruptor, cytochalasin B, because the initial cell count was determined after drug treatments were applied, but prior to the washes, there may have been a significant number of parasites that were unable to attach due to the presence of drugs, making the initial cell count significantly lower. Also, there may have been a significant number of parasites in which attachment was weakened or incomplete due to the presence of drugs, but appeared to have normal attachment. Typically, these parasites would be removed during the washes.

In contrast to Feely and Erlandsen (1982), Gillin and Reiner (1982) found no effect on *Giardia* attachment to a glass substrate in the presence of 10 and 25 mg/ml of cytochalasin-B, C, and D [202] [211]. Here, *Giardia* cultures were enumerated using a coulter counter to determine an initial cell count. The cultures
were incubated in borosilicate glass tubes at 35° C in MM2 media with either
cytochalasin B, C, or D (dissolved in DMSO and diluted in MM2) over a two-hour
time course. Nonattached parasites were removed by vacuum aspiration and the
tubes were rinsed with 1ml of warm media. The remaining attached cells were
removed by chilling for 10 minutes in 1ml of cold phosphate buffer (pH 7.2) 85%
saline solution. Samples were diluted in counting buffer and the percent attached
was determined using a coulter counter, which importantly, does not discriminate
between live and dead cells. Gillin and Reiner found no effect on Giardia
attachment to a glass substrate in the presence of 10 and 25 mg/ml of cytochalasin-
B (97 and 91% of initial count), cytochalasin-C (95 and 94% of initial count), or
cytochalasin-D (87 and 74% of initial count) during the two-hour time course.
Gillin and Reiner attributed the lack of cytochalasin effect to differences in Giardia
species, and the implemented techniques. In particularly, their technique does not
differentiate between living and dead cells. Furthermore, no positive control was
done to insure that either their techniques or their reagents were working properly.
Although they state that results for all experiments were “typical” of 2-5
experiments, it is not clear how many times an individual experiment or a subset of
experiments were repeated.

While studies by Feely and Erlandsen (1982), and Gillin and Reiner (1982)
served as a good starting point, it is important to mention that the aim of their
studies does not focus primarily on examining the effects of microtubule or microfilament disrupting drugs on attachment. Feely and Erlandsen (1982) also measured the effects of low Ca\(^{++}\) concentration, iodoacetic acid, and quinacrine-HCl on attachment [202]. They showed, using the same technique, that a low Ca\(^{++}\) concentration decreased attachment to 50.3\% after 40 minutes, while attachment in higher Ca\(^{++}\) concentrations remained high at 105 and 91.3\%, respectively. Given that the contractile active of actin requires free Ca\(^{++}\) (reviewed in [185]), Feely and Erlandsen (1982) suggested that the cytochalasin-B and low Ca\(^{++}\) concentration effects on parasite attachment support the role of contractile proteins in the mechanism of attachment [202]. Gillin and Reiner (1982) also measured several other parameters including, reducing agents, serum, temperature, and ion concentrations, including Ca\(^{++}\) [211]. However, unlike Feely and Erlandsen (1982), but in agreement with their study, they found that low Ca\(^{++}\) concentrations had no effect on attachment [202]. Overall, the discrepancy between these two studies is likely a result of the implemented techniques used to measure attachment, and perhaps due to low repetition of experiments. Additionally, the experiments were performed in different media, which may have interacted with the drugs.

To provide a more comprehensive investigation on the role of microtubules and microfilaments in attachment, we have performed \textit{in vitro} attachment assays in glass tubes using the microtubule disrupting drugs; nocodazole (2\,\mu M, 20\,\mu M, and
200uM), paclitaxel (2uM, 20uM, and 200uM), and colchicine (200uM and 1uM), and the microfilament disrupting drugs; cytochalasin-B (2uM, 20uM, and 200uM), cytochalasin-D (2uM, 20uM, and 200uM), and latrunculin-A (2uM and 20uM) (Khoury and Elmendorf, unpublished). Although previous drug studies have generated conflicting results, here, treatment of *Giardia* with nocodazole and paclitaxel failed to show a significant effect on attachment at concentrations below 200uM, while treatment of *Giardia* with colchicine had no effect on attachment, even at 5uM. Alternatively, treatment of *Giardia* with the microfilament disruptors, cytochalasin-B, cytochalasin-D, and latrunculin-A, demonstrated a significant dose dependent reduction in attachment. Significant reduction was observed as early as 30 minutes, even at a low concentration (2uM). Moreover, by 240 minutes attachment of cytochalasin-B (200uM) treated parasites was reduced by 60%, and latrunculin-A treated parasites was reduced to below 5% (2uM and 20uM). Given the stronger potency of cytochalasin-D (10 times that of cytochalasin-B), a more dramatic reduction in attachment was observed, as attachment was reduced to 20% (2uM) as early as 30 minutes, and as low as 5% (2uM) after 240 minutes. Remarkably, our results implicate an important role for microfilaments and not microtubules in attachment. These results correlate well with microscopy observations by Correa and Benchimol (2006) that show cytochalasins B and D had a profound effect on *Giardia* morphology and caused
parasites to detach from glass [209].

1.9. Drug Discovery: Targeting *Giardia* Actin

1.9.1 *Actin Microfilaments*

Traditionally, among crown organisms, actin is considered to be an abundant and highly conserved protein that participates in a plethora of protein-protein interactions, including interactions with other actins monomers (G-actin) to form polarized actin filaments (F-actin). The immediate assembly and disassembly of actin filaments are required for many cellular functions including, cell structure and polarity, cellular division, intracellular transport, and cell motility and muscle contraction. The polymerization, depolymerization, and stabilization of these filaments are tightly regulated by the hydrolysis of ATP-actin and an abundance of actin binding proteins (ABPs) that include, monomer binding proteins, capping proteins, small severing proteins, side-binding proteins, cross-linking proteins, membrane attachment proteins, and myosins (reviewed in [185] [186]).

The importance of the cytoskeleton in maintaining cellular structure and functions makes it an appealing drug target against eukaryotic pathogens. However, this approach is complicated by the fact that healthy non-pathogenic cells are also likely to be targeted, thus creating issues with toxicity. Remarkably, slight
variations in the sequence of a protein can be significant enough to result in slight structural modifications. Although the proteins may function similarly, these modifications, particularly at binding sites, can be significant enough to either inhibit or promote the preferential binding of small molecule drugs. Thus, proteins with subtle structural differences are often exploited as potential drug targets.

1.9.2 Targeting Actin

The crystallized structure of actin associated with the marine toxins, kabiramide C and Jaspisamide A has been determined and shown to bind the hydrophobic cleft between subdomains 1 and 3 [212]. These toxins are known to target actin with high affinity and specificity, and demonstrate strong filament severing and monomer sequestering properties [213] [214] [215]. Importantly, natural small molecule marine products, which potently target microfilaments with high affinity and specificity, have been shown to display cytotoxic effects against several types of multidrug resistant tumors, and therefore have made promising candidates for drug development against cancer cells [216] [217] [218] [219]. Additionally, it has been reported that microfilaments in the parasitic protists, Leishmania, have structural differences that influence the binding of small molecules, indicating its significance as a potential drug target [220].
1.9.3 Targeting Giardia Actin

Although actin is considered highly conserved among crown organisms, it is considerably less conserved than tubulin among the entire eukaryotic domain. *Giardia* actin, which was identified as a single copy gene by southern blot analysis, has a 58\% amino acid identity to other eukaryotic actin sequences [221]. Moreover, a bioinformatics investigation of the *Giardia* cytoskeleton concluded that tubulin and its associated proteins appeared to be notably well conserved when compared to *S. pombe*, *S. cerevisiae*, *C. elegans*, *Homo sapiens*, *Drosophila*, and *Chlamydomonas*, whereas actin, similar to what Drouin *et al.* (1995) found, was relatively less conserved [181] [221]. Specifically, bioinformatics showed that actin, and its associated proteins are noticeably underrepresented in *Giardia*. In particular, there appears to be an absence of numerous actin-associated proteins including, ARP1, APR2, ARP3, AFD/cofilin, profilin, thymosin β-4, gelsolin, myosin, α-actinin, filamin, villin, fimbrin, and formin [181].

These results are quite perplexing, given that these proteins are known to participate in a broad range of functions including, actin modification, organization, and assembly. A possible explanation for the lack of actin-associated proteins may be attributed to an unusual degree of sequence divergence from the more conventional actin-associated proteins. This would make detection by traditional BLAST searches difficult. Alternatively, *Giardia* may be missing the typical
eukaryotic microfilament system, and instead may possess novel proteins to perform traditional functions. Nonetheless, with such an elaborate cytoskeleton that is vital to the parasite’s survival, it is unlikely that Giardia is missing proteins necessary for the polymerization, modification, and/or the organization of actin microfilaments, and thus it is more plausible that either novel or divergent proteins substitute functionally for more conventional proteins.

The lack of traditional actin-binding proteins not only suggests that Giardia may have other novel or diverse protein that act as functional substitutes, but it also suggests that Giardia actin may have slight structural modifications that would enable more diverse or novel proteins to bind. To exemplify this, co-incubation experiments with Giardia and intestinal epithelial cells failed to localize Giardia actin with fluorescent-labeled phalloidin, which commonly binds to most actins. Importantly, intestinal epithelial cells showed characteristic F-actin staining (Khoury and Elmendorf, unpublished). To further exemplify this, in silico molecule docking of phalloidin to a putative 3-D model of Giardia actin predicted phalloidin to bind Giardia actin at a significantly lower affinity than human β-actin based on structural difference at the phalloidin-binding site (Rajnarayanan, Borges, Elmendorf, and Pattabiraman, unpublished).

Apart from Giardia, phalloidin also fails to bind to Tetrahymena actin. This has been attributed to amino acid substitutions at two crucial residues (Trp/Tyr at
Trp 79; Tyr/Leu at Tyr 198) [222] [223]. Although *Giardia* actin contains the conserved Trp at position 79, similar to *Tetrahymena*, it contains an amino acid substitution at position 198 (Tyr/Ala). Although these substitutions represent slight structural modifications, they are clearly enough to incite differential binding of a common actin-binding molecule.

The failure to localize *Giardia* actin with fluorescent-labeled phalloidin, in conjunction with the *in silico* predicted low affinity docking of phallolidin to a putative 3D model of *Giardia* actin suggests that, similar to *Leishmania* and *Tetrahymena*, *Giardia* actin may have structural differences that effect the binding of small molecules, thus promoting its usefulness as a potential drug target. Furthermore, multiple drug studies involving microfilament disrupting drugs have indicated a role for microfilaments in attachment, which is required for *Giardia* to establish and maintain an infection [202] [209] (Khoury and Elmendorf, unpublished). These findings imply that *Giardia* actin should be further investigated as a potential drug candidate.
CHAPTER 2

HIGH-THROUGHPUT SCREENING AND THE IDENTIFICATION OF SMALL MOLECULE COMPOUNDS THAT TARGET GIARDIA ATTACHMENT

ABSTRACT

*Giardia lamblia* is a prevalent intestinal parasite and cause of diarrheal disease throughout the world. Importantly, in order for parasites to maintain an infection they are required to attach to the epithelial cells in the small intestine. Although the mechanism of attachment remains elusive, its requirement in establishing and maintaining an infection makes it an appealing target for developing new chemotherapeutics. Additionally, newly identified drugs that specifically target the process of attachment could further be used as probes to identify the molecular machinery required for parasite attachment. In turn, understanding the mechanism of attachment will ultimately lead to the identification of both new and currently available chemotherapeutics.

Here, we have developed and implemented a high-throughput microscopy-based phenotype assay to screen 1,978 compounds included in the NCI Diversity Set I Library for their ability to block parasites attachment to an inert substrate. Our screen identified 88 compounds that strongly inhibited *Giardia* attachment (>80%)
and 141 compounds that moderately inhibited parasite attachment (50%-79%) during a two-hour co-incubation (50uM). Notably, our phenotypic Giardia attachment screen does discriminate between a direct ability for drugs to block parasite attachment, and a non-specific and indirect effect, such as cell death. To further evaluate our lead hits, we performed videomicroscopy to observe the morphological and behavioral changes of Giardia parasites exposed to our most promising compounds during an hour co-incubation. Abnormal phenotypes were categorized into one of three groups: parasites with disrupted morphology, parasites with normal morphology, but disrupted motility, and most relevant, parasites with normal morphology but unable to attach.

While the distinction of these phenotypes is irrelevant in identifying new chemotherapeutics, understanding the specificity of drug action for “non-attached” phenotypes is a critical step in determining compounds that will serve as tools in elucidating the mechanism and machinery of attachment. The significance of our top-town approach is two-fold; (1) identifying compounds that inhibit parasite attachment may be useful in identifying novel chemotherapeutic drug candidates and (2) a better understanding of the process of attachment will allow us to identify a larger range of potential drug targets, which are not currently being investigated.
2.1 Introduction

As one of the most prevalent intestinal parasitic infections worldwide, *Giardia* contributes significantly to the global burden of diarrheal diseases [224]. Infections are initiated through fecal-oral ingestion of infective cysts from contaminated drinking water or food supplies. Once ingested, the cysts pass through the stomach where excystation is triggered. The newly emerged trophozoites then enter the lumen of the small intestine where parasite replication and colonization occur [22]. A critical step in initiating and maintaining an infection is the ability of the trophozoites to attach themselves to the epithelial lining of the small intestine. Failure to do such would result in parasite clearance by peristalsis, and consequently a reduction of both infection and transmission.

Although the medical relevance of *Giardia* infections is apparent, it’s highly divergent structural features and unconventional molecular strategies, make *Giardia* an appealing organism to study. In particular, one of the most prominent and distinct features of *Giardia* is a large disk located on the anterior portion of the parasite’s ventral surface just above the plasma membrane [187] [188] [189] [199].

The current theories of attachment are centered around three principle concepts, (1) binding and/or adhesion at the molecular level, particularly lectin-mediated binding, (2) physical clutching – a cat’s claw model – focusing on the interaction of
the lateral crest of the ventral disk with the intestinal epithelial mucosa, and (3) negative pressure, the source of which is currently unknown (reviewed in [151] [195]).

A series of *in vitro* attachment/detachment studies over the past twenty years argues against a primary role for molecular interactions in attachment. *Giardia* attaches to plastic and glass in culture as effectively as it attaches to intestinal cell lines [200], and in a comprehensive study that quantified parasite attachment forces for the first time, *Giardia* detachment forces were determined to be equivalent, regardless of surface composition (charged, hydrophobic, or inert) [210]. And importantly, a recent study directly demonstrated that the adhesive activity of the ventral lateral flange alone could permit attachment in a small minority of parasites [204]. Collectively, these findings argue against a primary role for molecular interactions in parasite attachment, although lectin may assist in the process of attachment *in vivo* by facilitating a tight initial contact between the parasite and host mucosal surface.

Hence, many recent investigations have focused primarily on biomechanical forces as a mediator of attachment (reviewed in [151] [195]), and more specifically on the relative contributions of microtubules and microfilaments to the biomechanical forces [202] [202] [211] [38] [40] [59] [60]. Included among the many drugs used to examine the role of the cytoskeleton are the microtubule-
disrupting drugs, colchicine, paclitaxel, and nocodazole, and the microfilament-
disrupting drugs cytochalasin B and D, and latrunculin A. These studies have
demonstrated that the cytoskeleton is the primary mediator of attachment, although
there is still disagreement on the relative contributions of the microtubule and
microfilament network.

Because the process of attachment is a necessary step in the establishment of
infection, it presents itself as a possible target in the development of new
chemotherapeutics to treat giardiasis. The discovery and development of novel
drugs that specifically target the process of attachment would be a significant step
forward in determining the mechanism and additional cellular requirements for
parasite attachment. More importantly, a better understanding of the underlying
mechanism of attachment would ultimately lead to the identification of both new
and currently available drugs for the treatment of giardiasis. Drug development
efforts can proceed in either a “bottom-up” approach – starting with a known
protein target and identifying small molecule inhibitors of its function – or with a
“top-down” approach – identifying small molecule inhibitors of a process in the
absence of specific knowledge about machinery; indeed the small molecule
inhibitors identified in a “top-down” approach can then be used as probes to
discover components of the protein machinery involved.
Given our knowledge about the involvement of the cytoskeleton in attachment, one version of a “bottom-up” approach would be to use inhibitors of cytoskeletal function as starting points in drug development. However, all drugs that have been used in studies to date target both the host and parasite cytoskeleton and are therefore not candidates for novel chemotherapeutics. An alternative approach is represented in a recent study by Lauwaet and colleagues, in which known antimicrobials are used in a new system to determine their broader drug applicability; here the use of isoflavones, which act on a variety of pathways, was shown to cause rapid detachment of parasites and was recognized as a possible tool to further delineate the molecular mechanism underlying parasite attachment [225]. While successful, this approach is limited to known compounds.

Therefore, given our very limited understanding of the full mechanism of attachment, our approach in the work described here has been a “top-down” method using a phenotypic assay to screen a large library of small molecule inhibitors with the goal of simultaneously identifying potential new lead compounds to be used in the treatment of giardiasis and tools for the identification of the attachment machinery. To do this, we have developed a high throughput screening to assess *Giardia* attachment in response to 1,978 compounds included in the NCI Diversity Set I library, which was acquired from the NCI/DTP Open Chemical Repository (http://dtp.cancer.gov). From these *in vitro* efforts we have
identified seven lead compounds that are active against *Giardia* but that do not affect a rat intestinal epithelial cell line.

### 2.2 Materials and Methods

**Cell Cultures**

*Giardia lamblia* trophozoites (isolate WB1267) were maintained anaerobically in borosilicate glass tubes or polystyrene flasks (Fisher Scientific, 07-200-72). Parasites were grown at 37°C in modified TYI-S-33 media [226], where phosphate buffer solution was substituted with 0.024 M sodium bicarbonate. For all experiments, *Giardia* cultures were grown to mid-log phase (~80% confluency).

IEC-6 cells (ACCT CRL-1592) were cultured in DMEM media (Invitrogen, 10313-039) supplemented with 10% FBS (Gemini Bio-Products, 100-106), 4mM L-glutamine (Invitrogen, 25030-081), 0.1 unit/ml bovine insulin (Sigma 10516), and 1% pen-step (Fisher, 17-745E). IEC-6 monolayers were grown in culture tissue flask (Fisher Scientific, 10-126-34) at 37°C in 5% CO₂ and maintained at log phase (~80-90% confluent).
**Giardia Attachment Assays**

The Developmental Therapeutics Program at the National Cancer Institute in Bethesda, MD generously provided all small molecule compounds included in the NCI Diversity Set I library (1,978 total), which is part of the larger NCI/DTP Open Chemical Repository (http://dtp.cancer.gov). NCI drugs were dissolved in 100% DMSO (Sigma, D2650) to a final concentration of 0.5% DMSO for all assays. The Diversity Set 1 compounds were dispensed into 96-well flat-bottom polystyrene plates (Fisher Scientific, 07-200-91) at a final concentration of 50uM. To avoid edge effects, wells A1-H1 and A12-H12 were omitted, as observations indicated overall lower cell attachment in these wells in the absence of drug. As a result, all drug compounds were strategically placed in internal wells, while untreated controls were placed in the outer wells to avoid any bias in favor of drug-induced detachment. Thus, untreated controls were place vertically on the edges of each plate in wells A2-H2, and A11-H11. The average cell count for the 2 wells within a row (i.e. A2 and A11) were then averaged to determine the number of untreated cells for that row. Culture flasks containing approximately 5-8 X10^5 parasites/ml (~80% confluent) were chilled on ice for 20 minutes to induce detachment. Cells were centrifuged at 1015 x g for 5 minutes at 4°C and resuspended in one-half volume of ice-cold 1X PBS. 150ml of *Giardia* trophozoites (~100,000 parasites) were added to each well, followed by the
addition of individual drug. Parasites and drug were mixed by gentle pipetting and then incubated for 2 hours at 37°C in 5% CO₂. Wells were gently washed twice with warm 1XPBS (37°C) to remove unattached parasites. Cells were fixed at room temperature for 3-5 minutes in a 1:1 ratio of methanol and acetone and then stained for 20 minutes in 8µM Syto16 green fluorescent nucleic acid dye (Invitrogen, S7578). Plates were stored at 4°C.

Automated Imaging and Cell Counts

Images were captured on a Nikon Eclipse TE-300 inverted time-lapse microscope. Metamorph® version 7 screening module was used to perform automated imaging and cell counts. Four images per well were captured at 10X magnification, which together represent approximately 20% of each well. Automated cell counts were determined using parameters that measured the cell size and the signal to noise ratio. Attachment was calculated as a percentage of the untreated cells. Since cells were distributed using a multichannel pipettor, percent attachment was calculated on a row-by-row basis by comparing all cell counts for drug treatments to the untreated controls within the same row. This prevented any bias caused by potential pipetting errors. As additional controls, Giardia attachment was measured in response to relevant concentrations of DMSO (empty vehicle control) and mercury (NSC268879). The percent attachment for the initial
screening of all 1,978 Diversity Set 1 compounds was determined by using the median value of three trials.

IEC6 Attachment and Viability Assays

IEC-6 (ACCT CRL-1592) monolayers (~80-90% confluent) were washed with IX PBS and treated with 0.06% trypsin-EDTA (Invitrogen, 25200-056). Cells were centrifuged at 877 rpm, resuspended in 1X PBS (3-4 X 10^5 cells/ml), and distributed into 96-well plates (Fisher, 07-200-91) at 100 ml/well. To facilitate adequate cell attachment plates were incubated overnight at 37°C in 5% CO₂. To remove culture media plates were gently washed twice with warm 1X PBS (37°C). NCI Drugs were added to the wells at a final concentration of 50uM in 1XPBS and incubated for 2 hours at 37°C in 5% CO₂. The wells were gently washed twice with warm 1X HBSS (37°C) to remove drug and unattached cells. To stain for viability the cells were incubated at room temperature for 15 minutes in 2.6 uM EthD2 (Invitrogen, E3599). Cells were fixed in 4% glutaraldehyde (Sigma G-5882) for 10-15 minutes. Fixed cells were washed twice with 1X HBSS and stained in 8uM Syto16 green fluorescent nucleic acid dye (Invitrogen, S7578) for 20 minutes. Automated images were collected for each well using GFP and DsRed filters.
Percent attachment and viability were determined by comparing the number of attached cells and non-viable cells to that of non-treated cells. Since cells were distributed using a multichannel pipettor, percent attachment and viability was calculated on a row-by-row basis by comparing all cell counts for drug treatments to the untreated controls within the same row. This prevented any bias caused by potential pipetting errors. Results represent the median value of 9 trials.

Statistical Analysis

DMSO empty vehicle experiments were replicated 3-4 times with 4-6 samples per replicate. Mercury control experiments were done in duplicate with 6 samples per replicate. For both DMSO and mercury control experiments actual cell numbers were used to perform a single factor ANOVA for each individual concentration and relative control group.

Parametric analysis was performed using SAS 9.1.3 programming software (2000) to compare *Giardia* attachment in response to the top seven NCI drug candidates to that of untreated or mercury treated controls. P values were determined by performing a one-way ANOVA using a cubed root transformation of the actual cell numbers. A Tukey-Kramer adjustment was used to determine the adjusted P values for all parametric analysis. Data from the IEC-6 viability screening did not meet the assumptions for parametric analysis, therefore to
compare IEC-6 cell attachment in response to the top seven NCI compounds a non-parametric t-test was performed on cell numbers using SPSS 16.0 (2009) statistical programming software. The Mann-Whitney method was used to determine P values.

*Video Microscopy Observation of Giardia Morphology and Motility*

*Giardia* trophozoites were grown to log phase (~80% confluent). Cell cultures were decanted and media was replaced by ice-cold 1X PBS. Cultures were chilled on ice for 15 minutes to induce detachment and cells were collected by centrifugation at 2,500 rpm for 5 minutes. Parasites were resuspended in 1X PBS with the indicated drug at a final concentration of 50uM, 15uM, 10uM, 5uM, or 1uM. Cells were transferred into 10-well glass slides (Fisher,12-565-105) and incubated in a damp chamber in the presence of drug at 37°C and 5% CO₂ for 1 hour. A cover slide was placed over the slide and 5 seconds of video microscopy was recorded at 63X using DIC optics on a Zeiss Axioplan microscope and a Photometrics CCD camera (Table 2). Still frame images (Figure 3) represent 1 minute microscopy videos after 2 hours incubation with drug. Velocity software 5.3 (Imparvision) was used to capture video, which was converted to QuickTime 7 for analysis.
2.3 Results

Compounds Identified in Primary High Throughput Attachment Assay

We have developed a phenotype-based high-throughput screen of the 1,978 compounds in the NCI Diversity Set I to identify compounds that block the ability of the parasites to attach to an inert substrate. Because *Giardia* attaches with similar forces to a wide range of inert and biological substrates [210], we have conducted our primary screen in polystyrene microplates. Primary screening of the NCI Diversity Set I was performed in duplicate. For all attachment assays, *Giardia* trophozoites were dispensed into 96-well plates and incubated in the presence of the various compounds for two hours; plates were gently washed to remove unattached parasites and cells were fixed and stained for automated imaging and cell counts (Figure 1). Parasite attachment was calculated as a percentage of untreated cells from wells within the same row of the 96-well plate. For a negative control, parasite attachment in response to DMSO alone was measured. Concentrations below 1% had no significant effect on parasite attachment, in agreement with previously published data [227] (Figure 2A); likewise, in a positive control, attachment assays using 50uM-1.5uM mercury (NSC# 268879), levels that are known to be toxic to parasites, showed mercury to significantly inhibit parasite attachment over a wide range of concentrations (Figure 2B).
High Throughput screening of 1,978 NCI compounds identified a total of 229 small molecule drugs that rapidly inhibited *Giardia* attachment at a final concentration of 50uM (Table 1). Compounds that inhibited parasite attachment by \( \geq 50\% \) at 50uM during a two-hour incubation period for at least one of the two trials were grouped together and selected for a third round of screening (245 compounds).

The median value for all three individual screenings was used to identify and classify final lead compounds. Drugs that inhibited *Giardia* attachment by \( \geq 80\% \) were defined as compounds that strongly inhibited parasite attachment, while drugs that inhibited parasite attachment over a range between 79\%-50\% were defined as drugs that moderately inhibited parasite attachment. Of the 245 compounds included in the third round of screening, 88 were shown to strongly (\( \geq 80\% \)) inhibit parasite attachment, and 141 compounds were shown to moderately (50-79\%) inhibit parasite attachment. In all, high throughput attachment screening identified 229 compounds as lead hits, which corresponds to \( \sim 12\% \) of the original compounds included in the NCI Diversity Set I Compound Library (NCI/DTP Open Chemical Repository, [http://dtp.cancer.gov](http://dtp.cancer.gov)) (Table 1).
Although we refer to the phenotypic screen performed on the *Giardia* parasites as an “attachment” assay, it is clear that parasites will not attach for a variety of reasons, ranging from the very non-specific (compound results in parasite death) to the very specific (compound targets a protein directly involved in the attachment process). While this distinction is irrelevant in identifying new potential chemotherapeutics for the treatment of giardiasis, distinguishing among these underlying causes for a “non-attachment” phenotype is a critical step in determining which compounds will be useful tools in elucidating the mechanism and machinery of attachment. Therefore, we have performed video microscopy to observe the morphological and behavioral changes of *Giardia* parasites exposed to our most promising compounds across a range of concentrations (50uM, 15uM, 10uM, 5uM, and 1uM) during an hour incubation. Videos were recorded at 63X for 5 second using DIC optics. Still images shown here (Figure 3) represent the most typical morphologies/behaviors observed on at least three separate occasions.

General observations for all 7 compounds fell into 3 phenotypic categories (Table 2): compounds that disrupted parasite morphology, often resulting in parasite death and loss (NSC5857, NSC82892 and NSC658142); compounds that did not affect gross parasite morphology but that caused flagellar paralysis.
(NSC38737); and compounds that did not affect gross parasite morphology or activity but that prevented attachment (NSC47949, NSC44480, and NSC76026).

2.4 Discussion

As a critical step in their life cycle, *Giardia* parasites must attach to the mucosal epithelium that lines the small intestine to establish an infection, which in turn allows for parasite replication and the shedding of large numbers of cysts into the environment to enhance transmission. Due the persistent shedding of intestinal epithelial cells, parasite attachment/detachment continues throughout the course of infection [151] [195]. Given the importance of parasite attachment in the life cycle, as well as establishing and maintaining an infection, it is not surprising that *Giardia* has evolved a highly effective mode of attachment to guarantee success of the parasite. However, delineating this precise mechanism has proven to be complicated, and as a result, several competing theories have emerged, all of which have supporting, but not conclusive evidence.

Although lectins may assist in the process of attachment *in vivo* – evidence in favor of a “sticking” model, *in vitro* attachment of *Giardia* to a wide range of inert substrates of varying charge and hydrophobicity suggests that it is not a requirement [200] [210]. These findings argue that although molecular binding
and/or adhesion, may have a prominent role, it is more likely to serve as a secondary mechanism that aids in the recognition of more suitable cell substrates or proper cell orientation [205]. Likewise, while a “clutching” model of attachment is supported by ultrastructural and video microscopy studies showing a prominent contractile or grasping motion at the edges of the ventral disk, which appears to be deeply rooted within the epithelial cell layer [187] [188] [206] [45, 51] [204], it does not explain more recent findings, which showed a rapid decrease in parasite attachment in response to sharp changes in tonicity [228]. And a third negative pressure or “suction” model [203] [189] [188] [190] does not account for detachment, as observations demonstrate the ventral flagella beat continuously regardless of cell activity [151] [195].

The lack of consensus within the field regarding parasite attachment is due in part to a deficiency of sufficient research tools. Early studies that indicated the involvement of the cytoskeleton opened a new possible avenue of investigation, and indeed there is a small but interesting body of literature examining the effect of both microtubule-disrupting compounds and microfilament-disrupting compounds on parasite attachment and ultrastructure [151] [195]. The most recent studies seem to suggest a more prominent role for microfilaments – perhaps one of our current best leads in understanding the mechanism and machinery of attachment. However, further insights into microfilament function have been slowed by the
recent findings of the genome project that documented the absence of homologues for all known microfilament proteins other than actin [209] [202] [229]. While these striking differences in machinery increase the interest in the microfilament cytoskeleton as a potential drug target, it has thwarted efforts to study the attachment process at the molecular level.

Small molecule inhibitor screens have recently emerged as a promising means of identifying new compounds that, in altering a cellular behavior, can be used as a probe to identify the relevant molecular machinery: chemicals as “bait” in molecular fishing expeditions [230] [231]. While recent studies by Lauwaet and colleagues have used a deductive approach to identify isoflavones as a modulator of attachment and therefore a potential tool for the mechanistic identification [225], such focused studies are limited to a very narrow investigation and identification of future small molecule inhibitors as tools for discovery.

Here, instead, we have developed a high-throughput assay that measures the parasite attachment in response to 1,978 compounds that are part of the NCI diversity set small molecule library. This compound library is part of an “open repository” that consists of ~140, 000 compounds. These compounds were narrowed down to ~72,000 based on availability, and then further streamlined lined to 1,978 by Chem-X, to define compounds that represented the greatest pharmaocophore diversity.

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(http://dtp.nci.nih.gov/branches/dscb/div2_explanation.html). Thus, in a relatively small screen, an enormous diversity of chemical structures is evaluated. In turn, further analyses that pursue the larger family of structurally-related compounds can then extend the optimization of the inhibitors and probes.

One consequence of the use of a Diversity Set library is the recognition that the structural variant chosen from a family as the representative member of the set may not be the best inhibitor from that family. Thus it is necessary to conduct initial screens at relatively high concentrations and look more rigorously at dosage-dependent effects at later screening stages. High-throughput drug screens are also complicated by solubility issues: compounds that are highly soluble (hydrophilic) in aqueous solution may not be able to cross the phospholipids bilayer of the cell membrane, while compounds that are relatively insoluble (hydrophobic) and can cross cell membranes may readily precipitate out of solution and hence be unavailable in the bioassay.

With these constraints in mind, we have chosen to relax inclusion criteria in the first rounds of our high-throughput screen. Our initial screening of the NCI diversity set library (1,978 compounds) was done in duplicate at a relatively high concentration of 50uM to increase the sensitivity of the assay, and we have opted to include all compounds that showed efficacy in one of two trials (in the first screen)
and two of three trials (in the second screen). This maximized our dataset for future more detailed analysis.

In total, we identified 229 NCI compounds that inhibited *Giardia* attachment by $\geq 50\%$. These compounds represent $\sim 12\%$ of the NCI diversity set (Table 1). Of these compounds, 88 were identified as inhibiting parasite attachment $\geq 80\%$ and another 141 were identified as inhibiting parasite attachment between 79%-50%. These groups represent $\sim 4\%$ and $\sim 7\%$ of the NCI diversity set, respectively.

While our high-throughput screening was able to identify compounds that inhibited parasite attachment, it was not able to identify compounds that directly inhibited parasite attachment compared to compounds that induced detachment as result of parasite death. However, our screening was selectively done over a short two-hour incubation to screen for compounds that had an immediate or rapid effect. This allowed us to avoid drugs that specifically interfered with long-term cellular processes, such as cellular division, and target more immediate processes, such as attachment, morphology, motility, and parasite behavior (Table 2 and Figure 3).

To further investigate the mechanism of drug activity, further rounds of screening were conducted at a range of concentrations, to enhance our confidence that the observed effect was specific to the initial target of the drug. We screened
114 top lead candidates for their ability to inhibit parasite attachment at concentrations ranging between 50uM-1uM. A total of 7 compounds were shown to significantly inhibit parasite attachment at 10uM, and five of these were shown to significantly inhibit parasite attachment at 5uM (see chapter 3; 3.5, Figure 3).

We have further conducted qualitative observations of parasite behavior and morphology to give more insight about the drug target. Observations of parasites exposed to relevant concentrations of drug (50uM-1uM) resulted in the identification of 3 broad phenotypes (Table 2). Among these phenotypes, four compounds resulted in severe disrupted morphology and in some cases, cell lysis. However, more interestingly, we have observed two phenotypes, in which morphology does not appear to be significantly disrupted and drug effect seems to be more targeted towards either motility (NSC37837) or behavior (i.e. attachment) (NSC47949, NSC44480 and NSC76026). Of these compounds, NSC37837 and NSC76026 were also shown to be at least partially reversible (data not shown), which is consistent with targets of the cytoskeleton or other adapter proteins. Although these observations are only qualitative, and need to be further investigated, they are an important first step towards identifying potential drug targets.

While our identified lead compound hits will need further analysis, it is a significant step towards identifying new tools that may begin to unravel the precise
mechanisms by which *Giardia* is able to achieve and regulate attachment. The importance of this is two-fold; (1) identifying compounds that inhibit parasite attachment may be useful in identifying and developing novel drug candidates for the treatment of giardiasis (see chapter 3), and (2) a better understanding of the process of parasite attachment itself will allow us to identify a larger range of potential drug targets, which are not currently being investigated.
2.5 Figures and Tables

*Giardia* High-Throughput Attachment Screen

Figure 1. *Giardia* High-Throughput Attachment Screen. *Giardia lamblia* attachment was evaluated in response to 1,978 small molecule compounds from the NCI diversity set library. Parasites were transferred into 96-well plates and incubated in the presence of drug at 50uM for two hours. Wells were gently washed to remove unattached parasites. Cells were fixed and stain with a fluorescent nucleic acid dye for visualization. Metamorph™ HTS programming software was used to perform automated imaging and cell counts. A total of 4 images per well were captured representing ~20% of each well. *Giardia* attachment was calculated as a percentage of untreated cells.
Empty Vehicle and Mercury Controls

Figure 2. Empty Vehicle and Mercury Controls. Values represent the mean percent attachment and error bars represent the SEM. A) All NCI diversity sets drugs were dissolved in 100% DMSO. Final DMSO concentration for all assays was 0.5%. Relevant concentrations of DMSO (≤ 1μM) had no significant effect on Giardia attachment (p > 0.05) (*p = 0.035). B) Attachment assays with mercury (NSC# 268879) demonstrated a significant effect on parasite attachment between 50uM-1.5uM (p value <0.05 for all mercury concentrations).
NCI Compounds Identified in *Giardia lamblia* High-Throughput Attachment Screen

<table>
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<th>NCI Compounds that <em>Strongly</em> Inhibit <em>Giardia</em> Attachment</th>
<th>NCI Compounds that <strong>Moderately</strong> Inhibit <em>Giardia</em> Attachment</th>
<th>Total NCI Hits</th>
<th>Total Number of NCI Compounds</th>
</tr>
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<tbody>
<tr>
<td>88 (~4%)</td>
<td>141 (~7%)</td>
<td>229 (~12%)</td>
<td>1,978</td>
</tr>
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</table>

Table 1. NCI Compounds Identified in *Giardia lamblia* High-Throughput Attachment Screen.

Parasites were incubated in the presence of drug for 2 hours at 50μM. Cells were fixed and stained, and automated imaging and cell counts were performed to determine the percent of attachment relative to controls. Attachment values represent the median of three independent trials (*Giardia* attachment inhibited ≥80%, **Giardia** attachment inhibited between 50%-79%). Of the 1,978 diversity set compound 229 drugs inhibited *Giardia* attachment ≥50%, which represents ~12% of the NCI diversity set small compound library.
Effect of Lead Compounds on Parasite Morphology and Motility

Figure 3. Effect of Lead Compounds on Parasite Morphology and Motility. Video microscopy was used to assess the morphology and motility of *Giardia* parasites incubated in the presence of our seven top lead candidates (10μM) for two hours. A single frame from each 1-minute video is shown for each compound. (A) untreated control cells; (B) NCS5857 (C) NCS38737 (D) NCS44480 (E) NCS47949 (F) NCS658142 (G) NCS76026 (H) NCS82892. Unattached cells out of the plane of focus are seen as dark, blurry objects in the frame (Helou and Elmendorf, unpublished).
Table 2. *Giardia* Morphology and Motility Phenotypes. *Giardia* morphology and motility was observed in response to the top seven NCI drug candidates using video microscopy. Parasites were incubated in the presence of drug for one hour and video was taken for drug concentrations ranging between 50uM-1uM. Notable effects on either morphology or motility was observed for all drugs ranging between 50uM-10uM (+), and more mild effects were observed on 5 of the NCI drugs at 5uM (++) . A single compound, NSC658142 also showed noticeable disrupted morphology at 1uM (+++). Abnormal phenotypes were categorized into one of three groups: parasites with disrupted morphology, parasites with normal morphology, but disrupted motility, and parasites with normal morphology but unable to attach (Helou and Elmendorf, unpublished).
CHAPTER 3

IDENTIFICATION OF NOVEL DRUG CANDIDATES THAT TARGET THE PROCESS OF PARASITE ATTACHMENT IN GIARDIA LAMBLIA PATHOGENESIS

ABSTRACT

The intestinal parasite, *Giardia lamblia*, contributes significantly to the global burden of diarrheal diseases. The ability of parasites to initiate and maintain an infection is dependent on the parasites’ ability to attach to epithelial cells lining the small intestine. Furthermore, numerous studies have associated intestinal dysfunction with parasite attachment. Although the exact mechanism of *Giardia* attachment is unknown, its importance in pathogenesis, and in initiating and maintaining an infection are well established.

Metronidazole is the most common treatment for giardiasis. However, challenges associated with efficacy, high reoccurrence rates, and incidences of drug resistance have been reported. While alternative drug therapies exist, similar to metronidazole, they are not always effective or well-tolerated, which often leads to low compliance. These current treatment failures are cause for concern, and clearly indicate a need to develop novel drug therapies.
Unfortunately, most drug treatments for giardiasis have been identified by chance, and therefore may not be the most effective or efficient treatments. Hence, to identify novel, effective drugs that target *Giardia*, we developed a high-throughput phenotypic screen that targets *Giardia* attachment and viability. Previous screening of the NCI Diversity Set I library (1,978 compounds) identified 229 compounds that inhibit parasite attachment by at least 50%. Here, we developed a similar high-throughput assay using an IEC-6 rat intestinal cell line to examine the effect of compounds identified in our primary screening on host cell attachment and viability. In all, we identified 117 lead compounds that inhibited *Giardia* attachment by ≥50%, but only had a minimum to moderate effect on host cell attachment and viability. To further define potential lead compounds, we extended our analysis of top drug candidates to determine their ability to inhibit *Giardia* attachment over a range of concentrations (50μM-1μM). From our dose-dependent screening, we identified seven lead compounds that significantly inhibited parasites attachment at 10μM or less, without affecting host cell attachment and viability at a 5-10X higher concentration (50μM). Currently these compounds are under review for U.S. patent rights as prospective lead compounds in chemotherapeutic development to treat giardiasis (U.S. Provisional Application No. 61/266,660, Georgetown Ref. No.: 2009-016).
3.1 Introduction

*Giardia lamblia* is an intestinal parasite and predominant cause of diarrheal disease throughout the world. Although many individuals are often asymptomatic, giardiasis can be characterized by gastrointestinal symptoms such as diarrhea, severe abdominal cramping, nausea or vomiting, malabsorption, fatigue, and weight loss [1] [2] [232].

There are two distinct stages of the parasite’s life cycle. The infective cysts are metabolically quiescent and extremely resistant to environmental stresses, while the trophozoites are the metabolically active form of the parasite. Once ingested, excystation is triggered in the acidic and enzymatically-active environment of the stomach, and the newly emerged trophozoites enter the lumen of the small intestine where replication and colonization occur. To avoid clearance by peristalsis, and thus establish an infection, *Giardia* parasites must attach to epithelial cells lining the small intestine [22]. Moreover, due to the continuous shedding of intestinal cells, parasites must be capable of detaching, swimming, and reattaching to new sites within the small intestine [151]. Parasites that are eventually swept downstream of the small intestine are subject to changes in bile and lipid conditions, which triggers encystation, resulting in excretion of cysts [22] [23] [233].
Although the mode of pathogenesis remains unclear, numerous studies have associated intestinal dysfunction with parasite attachment. In particular, intestinal malabsorption and hypersecretion are thought to be the primary causes of diarrhea in *Giardia* infections [46] [61]. Parasite attachment to the intestinal epithelial layer damages microvilli, interfering with nutrient absorption [35]. Moreover, rapid parasite colonization can generate a physical barrier between the enterocytes and the intestinal lumen, thereby reducing the intestinal absorptive surface area, which further interferes with nutrient uptake. The enterocytic injury associated with parasite attachment leads to villus atrophy, intestinal hyperpermeability, and brush border damage [57] [35]. While the exact mechanism of parasite attachment is unknown, and remains a central point of interest, its importance in pathogenesis, and in establishing and maintaining an infection are abundantly clear.

Globally, diarrheal diseases cause nearly 4% of all deaths, and aside from respiratory illness, is the leading cause of death among children under 5 years [224]. The vast majority of these deaths occur in developing countries that lack adequate sanitation and clean drinking water [11]. Recent estimates by the World Health Organization indicate that 3.5 billion people worldwide live without clean drinking water or proper sewage [234] [11], and of these individuals, up to 1 billion may be infected with *Giardia* [10]. Importantly, while many infections are self-limiting, individuals frequently suffer from chronic infections, which can result in
an overall impaired state of health, as well as long-term growth retardation and impaired cognitive development [235] [236]. Given the propensity to cause widespread morbidity, *Giardia* has been added to the WHO Neglected Disease Initiative, which targets diseases that impair social-economic and developmental growth, and thus promote poverty [21].

Within the U.S., metronidazole, which targets the parasite’s anaerobic metabolism, is the most common treatment for giardiasis. However, it is typically less than 90% effective, and high rates of reoccurrence, and incidences of drug resistance have been reported. Although alternative drug therapies exist, including nitazoxanide, tinidazole (a nitromidazole), furazolidone (a nitrofuran), and albendazole (a benzimidazole), they are not always effective, or well-tolerated, which often leads to low compliance. Other challenges associated with current treatments include, severe side effects, especially with tinidazole [112], and for many drugs, contraindications [237] occur in AIDS patients or pregnant women [103] [112] [104] [105] [106]. Growing concerns regarding current treatment failures clearly indicate a need to develop novel drug therapies.

Historically, much of drug discovery in *Giardia* has occurred by chance. In particular, quinacrine, one of the first - and for many years only - treatment for giardiasis, was originally used to treat malaria [101]. Challenges associated with current drug treatments and the lack of *Giardia*-targeted large-scale drug discovery
has raised concerns of whether current drug therapies are the most efficient or
effective means for treating giardiasis. This deficit has now been recognized by the
field, and recently a large-scale drug screening was performed as a means to
identify compounds that inhibit Giardia growth [238]. The success of this research
supports the utility of high-throughput experimental approaches as an approach to
new drug discovery efforts in Giardia.

We have therefore developed a high-throughput phenotypic screen to identify
novel, effective and specific drugs that target Giardia viability and attachment
(Walls, et al., manuscript in preparation). Previously, we performed a high-
throughput screening of 1,978 compounds included in the NCI Diversity Set I small
Our results identified a total of 229 compounds that inhibit parasite attachment by
at least 50%. Here, we describe and report on a similar high-throughput assay that
examines the effect of the compounds identified in our primary screening on host
cell attachment and viability. In an effort to further define potential lead
compounds for novel drug candidates for the treatment of giardiasis, we extended
our analysis of the top drug candidates to determine their ability to inhibit Giardia
attachment over a range of concentrations and visually examined their physical
phenotype in the presence of drug (see chapter 2).
3.2 Materials and Methods

Cell Cultures

*Giardia lamblia* trophozoites (isolate WB1267) were maintained anaerobically in borosilicate tubes or polystyrene flasks (Fisher Scientific, 07-200-72). Parasites were grown at 37°C in modified TYI-S-33 media [226], where phosphate buffer solution was substituted with 0.024 M sodium bicarbonate. For all experiments *Giardia* cultures were grown to log phase (~80% confluent).

IEC-6 rat intestinal epithelial cells (ACCT CRL-1592) were cultured in DMEM media (Invitrogen, 10313-039) supplemented with 10% FBS (Gemini Bio-Products, 100-106), 4mM L-glutamine (Invitrogen, 25030-081), 0.1 unit/ml bovine insulin (Sigma 10516), and 1% penicillin-streptomycin (Fisher, 17-745E). IEC-6 monolayers were grown in tissue culture flasks (Fisher Scientific, 10-126-34) at 37°C in 5% CO₂ and maintained at log phase (~80-90% confluent).

Giardia Attachment Assays

The Developmental Therapeutics Program at the National Cancer Institute in Bethesda, MD generously provided all small molecule compounds included in the NCI Diversity Set I library (1,978 total), which is part of the larger NCI/DTP Open Chemical Repository (http://dtp.cancer.gov). NCI drugs were dissolved in
100% DMSO (Sigma, D2650) to a final concentration of 0.5% DMSO for all assays. The Diversity Set 1 compounds were dispensed into 96-well flat-bottom polystyrene plates (Fisher Scientific, 07-200-91) at a final concentration of 50uM. To avoid edge effects, wells A1-H1 and A12-H12 were omitted, as observations indicated overall lower cell attachment in these wells in the absence of drug. As a result, all drug compounds were strategically placed in internal wells, while untreated controls were placed in the outer wells to avoid any bias in favor of drug-induced detachment. Thus, untreated controls were placed vertically on the edges of each plate in wells A2-H2, and A11-H11. The average cell count for the 2 wells within a row (i.e. A2 and A11) were then averaged to determine the number of untreated cells for that row.

Culture flasks containing approximately 5-8 X10^5 parasites/ ml (~80% confluent) were chilled on ice for 20 minutes to induce detachment. Cells were centrifuged at 1015 x g for 5 minutes at 4°C and resuspended in one-half volume of ice-cold 1X PBS. 150ml of *Giardia* trophozoites (~100,000 parasites) were added to each well, followed by the addition of individual drug. Parasites and drug were mixed by gentle pipetting and then incubated for 2 hours at 37°C in 5% CO_2. Wells were gently washed twice with warm 1XPBS (37°C) to remove unattached parasites. Cells were fixed at room temperature for 3-5 minutes in a 1:1 ratio of
methanol and acetone and then stained for 20 minutes in 8\textmu M Syto16 green fluorescent nucleic acid dye (Invitrogen, S7578). Plates were stored at 4\textdegree C.

*Automated Imaging and Cell Counts*

Images were captured on a Nikon Eclipse TE-300 inverted time-lapse microscope. Metamorph® version 7 screening module was used to perform automated imaging and cell counts. Four images per well were captured at 10X, which collectively represent approximately 20\% of each well. Automated cell counts were determined using parameters that measured the cell size and the signal to noise ratio. Attachment was calculated as a percentage of the untreated cells.

Since cells were distributed using a multichannel pipettor, percent attachment was calculated on a row-by-row basis by comparing all cell counts for drug treatments to the untreated controls within the same row. This prevented any bias caused by potential pipetting errors. As additional controls *Giardia* attachment was measured in response to relevant concentrations of DMSO (1.5\%, 1.0\%, 0.5\%, 0.25\%, 0.125\%, 0.06\%) and relevant concentrations of mercury (50\textmu M, 25\textmu M, 12.5\textmu M, 6\textmu M, 3\textmu M, 1.5\textmu M) (NSC268879). The attachment values for the top seven drug candidates represent the median value of 5-9 trials.
IEC6 Attachment and Viability Assays

IEC-6 (ACCT CRL-1592) monolayers (~80-90% confluent) were washed with IX PBS and treated with 0.06% trypsin-EDTA for 5 minutes (Invitrogen, 25200-056). Cells were centrifuged at 125x g, resuspended in 1X PBS (3-4 X 10^5 cells/ ml), and distributed into 96-well flat-bottom tissue culture plates (Fisher, 07-200-91) at 100 ml/ well. To ensure adequate IEC-6 cell attachment, plates were incubated overnight at 37^0C in 5% CO2. To remove culture media plates were gently washed twice with warm 1X PBS at 37^0C. The selected NCI Diversity Set I compounds were added to the wells at a final concentration of 50uM in 1XPBS and incubated with IEC-6 cells for 2 hours at 37^0C in 5% CO2. The wells were gently washed twice with warm 1X HBSS at 37^0C to remove drug and unattached cells. To stain for viability, the cells were incubated at room temperature for 15 minutes in 2.6uM ethidium homodimer (Invitrogen, E3599). Cells were fixed in 4% glutaraldehyde (Sigma G-5882) for 10-15 minutes at room temperature. Fixed cells were washed twice with 1X HBSS and stained in 8uM Syto16 green fluorescent nucleic acid dye (Invitrogen, S7578) for 20 minutes at room temperature. Automated images were collected for each well using GFP and DsRed filters.

Percent attachment and viability were determined by comparing the number of attached cells and non-viable cells to that of non-treated cells. Since cells were distributed using a multichannel pipettor, percent attachment and viability was
calculated on a row-by-row basis by comparing all cell counts for drug treatments to the untreated controls within the same row. This prevented any bias caused by potential pipetting errors. Results represent the median value of 9 trials.

Statistical Analysis

Parametric analysis was performed using SAS 9.1.3 programming software (2000) to compare Giardia attachment in response to the top seven NCI drug candidates to that of untreated or mercury treated controls. P values were determined by performing a one-way ANOVA using a cubed root transformation of the actual cell numbers. A Tukey-Kramer adjustment was used to determine the adjusted P values for all parametric analysis. Data from the IEC-6 viability screening did not meet the assumptions for parametric analysis, therefore to compare IEC-6 cell attachment in response to the top seven NCI compounds a non-parametric t-test was performed on cell numbers using SPSS 16.0 (2009) statistical programming software. The Mann-Whitney method 1 was used to determine P values.
3.3 Results

Attachment and Viability Screening of IEC-6 Host Cells

Among the important criteria in drug development is selectivity; i.e. that the concentration at which the drug is effective against the pathogen be significantly different from the concentration at which it adversely affects host cells. Previous high throughput screening of the NCI diversity set small molecule library (1,978 compounds) identified 229 compounds that significantly inhibited *Giardia* attachment by $\geq 50\%$ at concentrations of 15uM or less (Walls, et al., manuscript in preparation). In addition to the 229 previously identified compounds, an additional 11 compounds, which were shown to inhibit *Giardia* attachment slightly less than 50% were also included (data not shown).

To evaluate the potential of these 240 NCI compounds as novel drug candidates for the treatment of giardiasis, a similar high-throughput screening was performed to access their effect on host cell attachment and viability. We used IEC-6 rat intestinal epithelial cells as a close representative of the cells that would be most exposed to the drug compounds during treatment. The IEC-6 cells were distributed into 96-well plates and incubated in the presence of each compound at a final concentration of 50uM for 2 hours. Wells were gently washed to remove unattached cells, and the remaining cells were stained with ethidium homodimer to evaluate host cell viability. Cells were then fixed and further stained with Syto-16.
nucleic acid dye to visualize total cell attachment relative to untreated controls. Automated imaging and cell counts were performed and viability was determined by comparing the number of attached cells and non-viable cells to that of controls (Figure 1). This screening was performed in duplicate for statistical validity of our analysis.

In total, 117 NCI compounds were identified as only weakly or moderately affecting host cell attachment or viability (Table 1). 85 NCI compounds were identified as having a minimal effect on IEC-6 cell attachment and viability (≥80% attachment and viability for both trials). An additional 33 compounds were defined as only moderately affecting host cell viability and attachment (50-79% attachment and viability for both trials). Of these 117 lead compounds, 114 (due to availability) were selected for further rounds of IEC-6 attachment and viability screening. This represents ~6% of the complete NCI diversity set small molecule library and ~48% of the NCI compounds included in the IEC-6 host cell attachment and viability screening.

Pooled data from previous Giardia attachment screening and IEC-6 cell attachment and viability screening identified four categories of potential drug candidates (Figure 2). The top lead group consisted of 19 NCI compounds that strongly inhibited Giardia attachment (≥80%), while only weakly affecting IEC-6 host cell attachment and viability (attachment and viability≥80%). Two other
groups of potential drug candidates consisted of 56 NCI compounds that moderately inhibited parasite attachment (79%-50%), while only weakly affecting host cell attachment and viability (attachment and viability ≥80%), and 10 NCI compounds that strongly inhibited parasite attachment (≥80%), while moderately affecting host cell attachment and viability (attachment 79%-50% and viability 70%-59%). A fourth group of potential drug candidates consisted of 21 drugs that moderately inhibited *Giardia* attachment (79%-50%) and moderately affected host cell attachment and viability (attachment 79%-50% and viability 70%-59%).

While no differential drug effect between *Giardia* attachment and host cell attachment and viability was identified in this fourth category of potential drug candidates, it is important note that differential effects may be observed at lower drug concentrations, and thus warrants further assessment. In total, 106 NCI compounds were identified as potential drug candidates for the treatment of giardiasis. This represents ~5% of the complete NCI diversity set small molecule library and ~44% of the NCI compounds included in the IEC-6 host cell attachment and viability screening.

*Dose Dependant Drug Curves and Identification of Final 7 Drug Candidates*

To further evaluate potential drug candidates for the treatment of giardiasis, NCI compounds that were identified as having a weak or moderate affect on IEC-6
host cell attachment and viability (114 compounds due to availability) (Table 1) were examined for their ability to inhibit *Giardia* attachment in a dose-dependant manner. Parasite attachment was measured by incubating non-attached parasites in the presence of drug for two hours at a range of concentrations (50uM, 15uM, 10uM, 5uM, and 1uM). Typically parasite attachment occurs within a very short period of time (5-10 minutes). Parasites that were unable to attach or unable to maintain attachment were removed by gentle washes, and the remaining attached cells were fixed and stained for visualization. Automated imaging and cell counts were performed and attachment was measured as a percentage of untreated controls. As a comparison, mercury (NSC268879), which is known to be toxic to *Giardia*, was used as an additional control. The median value of 5-9 samples was used to represent the percentage of parasite attachment, and actual cell counts were used to calculate adjusted P values.

Of the 114 NCI compounds that either weakly or moderately inhibited host cell attachment and viability (Table 1), 7 compounds were identified as significantly (Adj P value <0.05) inhibiting *Giardia* attachment at concentrations as low as 10uM (NSC5857, NSC38737, NSC44480, NSC47949, NSC76026, NSC82892, and NSC658142). Notably, IEC-6 host cell attachment and viability in response to a 2 hour co-incubation with these 7 drugs (50uM) was not significantly different (Adj P value <0.05) when compared to untreated controls (Figure 4).
these top seven drug candidates, median *Giardia* attachment values ranged between 2%-11% at 50uM, 3%-23% at 15uM, and 3%-15% at 10uM (Adj P value < 0.0001) (Figure 3). These results are consistent with mercury controls (NSC268879), which significantly inhibited parasite attachment (88%-80%) for drug concentrations ranging between 50uM-5uM. Five of the top seven candidates (NSC5857, NSC38737, NSC47949, NSC82892, and NSC658142) were also shown to significantly inhibit *Giardia* attachment at 5uM, as median attachment values for these compounds ranged between 1%-16% (Adj P value <0.05). While NSC44480 (Adj P value = 0.9116) and NSC760265 (Adj P value = 0.4251) did not significantly inhibit parasite attachment at 5uM, it is worth noting that median attachment values for these compounds ranged between 30%-47%, which was not significantly different than that of the mercury controls at the same concentration (Adj P value = 0.1242 and Adj P value = 0.5138, respectively). A probable explanation for the lack of statistical significance for these compounds is likely due to the large number of comparisons evaluated within a substantial data set (observations= 935), as non-adjusted P values were noticeably lower (P= 0.0140 and P= 0.0018). Furthermore, while no compounds were identified as significantly inhibiting parasite attachment at 1uM, median attachment values for two of the seven final candidates (NSC47949 and NSC658142) ranged from 44%-62%, which was considerably less than that of the untreated controls.
3.4 Discussion

Historically, many of the chemotherapeutic treatments for giardiasis have been discovered by chance or adapted for use against *Giardia* after successful treatment of other infections. For example, quinacrine, one of the first and for many years only treatment for giardiasis, was originally used to treat malaria [101]. Likewise, the current drug of choice in the U.S., metronidazole, is actually still used “off-label” for giardiasis and was originally developed in 1959 to treat trichomoniasis. The lack of *Giardia* targeted drug design raises the question of whether current drug therapies are the most efficient and/or effective means for treating giardiasis. Undesirable and potentially harmful side effects, which frequently lead to noncompliance, and high rates of reoccurrence, suggest otherwise [103] [104] [101].

Previously, we developed a phenotypic assay for *Giardia* attachment, which enabled us to conduct a high-throughput small molecule inhibitor screen of the 1,978 compounds that make up the NCI diversity set small molecule library (Walls, et al, manuscript in preparation) (see chapter 2). While the primary focus of this screen was to identify novel compounds that targeted the parasite’s ability to attach to an inert substrate – an important first step in developing new tools to investigate the mechanism and machinery of attachment – this screen also served the dual...
purpose of identifying compounds that were highly effective against the parasite as possible new leads in drug development efforts. Although to date, this is the only known high-throughput attachment assay used to identify potential drug candidates against *Giardia* attachment, a recent high-throughput screen, which identified compounds that inhibited *Giardia* growth [238] provides useful evidence regarding the feasibility of this approach in *Giardia* [105] [106].

To further evaluate compounds from the NCI Diversity Set I library as potential drug candidates for the treatment of giardiasis, we screened a set of 240 compounds identified as “moderately or highly effective” against *Giardia* in an attachment and viability assay against rat intestinal epithelial cells. While non-viable *Giardia* cells are unable to attach [202] [40] [60], it was unclear whether non-viable IEC-6 cells could potentially remain bound to the surface, especially in light of the fact that IEC-6 cells grow in monolayers. Therefore, to determine viability we first measured the percentage of attached IEC-6 cells and then compared the percentage of attached and non-viable cells to that of untreated controls. In general, we found that similar to *Giardia*, non-viable IEC-6 cells did not remain attached to the surface (Figure 4).

In total, our IEC-6 viability screen identified 117 lead hits, which were further evaluated for their ability to inhibit *Giardia* attachment in a dose-dependent manner (Figure 3). To further narrow the list of top lead candidates, we evaluated
all compound hits identified in our primary IEC-6 screen (Table 1) for their ability to inhibit parasite attachment at concentrations ranging between 50uM-1uM. While many of these compounds have already been shown to have a more measurable effect on *Giardia* attachment compared to that of host cells, top drug candidates ideally should have a high therapeutic index, which is defined as the ratio of the lethal dose for 50% of the population to the minimum effective dose for 50% of the population (LD$_{50}$ / ED$_{50}$). If the therapeutic index is adequately high then issues of toxicity become less relevant [239]. Our dose-dependent screen identified a total of 7 compounds that significantly inhibited parasite attachment at 10uM, five of which were also shown to significantly inhibit parasite attachment at 5uM (Figure 3). Although we did not determine LD$_{50}$ values for IEC-6 cell populations, we have sufficiently identified at least 5 candidates that have significant effects on parasite attachment at 1/10 the concentration at which IEC-6 cells attachment and viability was not significantly inhibited (Figure 3 and 4). It is also worth noting that although NSC44480 and NSC760265 did not significantly inhibit parasite attachment at 5uM, attachment values for these compounds ranged between 30%-47%, which was not significantly different than that of the mercury controls (see chapter 2, Figure 2B) at the same concentration. In all, we have identified 7 NCI drug candidates that significantly inhibit *Giardia* attachment at concentration as low as 10uM or 5uM, without affecting host cell viability at a much higher
concentrations (50uM). While these drugs are in a preliminary stage of development and further analysis is need to determine toxicity and the long-term affects on host cell viability, we have currently filed and are under review for a U.S. patent (U.S. Provisional Application No. 61/266,660, Georgetown Ref. No.: 2009-016).

Aside from measuring drug effect on parasite attachment in comparison to a mammalian host cell line, we also performed qualitative preliminary experiments to measure drug reversibility (Helou and Elmendorf, unpublished) (data not shown). After a two-hour incubation at 15uM, 4 of the top compound hits appeared to be largely irreversible (NSC5857, NSC44480, NSC47949, NSC658142, and NSC82892), while 2 showed at least a partial recovery, as parasites were able to reattach to glass after being washed (NSC38737 and NSC76026). Although compounds that exhibit rapid reversibility may not ultimately make a good candidate for clinical treatment, these compounds are still important, as they may be useful tools for investigating the underlying molecular mechanism of parasite attachment, which ultimately would lead to the identification of novel drug targets.

Other important considerations for the identification and development of novel drugs rely heavily on a compound’s ability to be absorbed and metabolized in a biological system. In particular, Lipinski’s rule of 5, which describes the biochemical properties that are important for the adsorption, distribution,
metabolism, and excretion (ADME) of a compound once it has entered into a human subject [240], is often used to evaluate the “druglikeness” or bioavailability of a compound. In general, according to Lipinski’s rule of 5, in order for a compound to be orally administered it may not violate more than one of the following criteria: (1) no more than 5 hydrogen bond donors (2) no more than 10 hydrogen acceptors, (3) it must have a molecular weight \( \leq 500 \) daltons, and (4) it must have an octanol-water partition coefficient \( \log P \) of less than 5 [240]. These criteria often come into play during drug optimization of a compound lead. Importantly, 3 of our 7 top compound leads (NSC38737, NSC76026, and NSC658142) already meet Lipinski’s rule of five, and therefore make promising drug candidates (Table 2).

While we have identified seven novel drug candidates, it is not likely they will all go forward into development. Foremost, more in-depth toxicity screens, including long-term viability assays need to be completed. We are currently in the process of doing this, and thus far preliminary evidence based on qualitative observation (Helou and Elmendorf, unpublished) has suggested three (NSC44480, NSC5857, and NSC82892) of our seven candidates may have toxic effects on host IEC-6 cells at 15\( \mu \)M during long-term incubations (2, 6, and 24 hours). However, we did not see the same effect at 5\( \mu \)M, in which only NSC82892 appeared to cause cell death, nor did we observe cell death for any of the drugs at 1\( \mu \)M. So while
NSC5857 appeared to induce host cell death at 15uM in our preliminary long-term study, it did not appear to cause host cell death for the same duration of time at a lower concentration of 5uM. Thus, NSC5857 remains a promising drug candidate, as it was found to significantly inhibit *Giardia* attachment at 5uM.

Other considerations include Lipinski’s rule of five (Table 2). Importantly, three of our seven compounds meet these requirements, including, NSC38738, NSC658142, and NSC76026. Of these three compounds both NSC38738 and NSC658142 were found to significantly inhibit parasite attachment at 5uM, but based on our preliminary qualitative observations didn’t appear to affect host cell viability during a longer incubation, even at 15uM. While there is still much to do to evaluate these drug candidates, particularly with toxicity, our current most promising drug candidates for the treatment of giardiasis are NSC38738 and NSC658142, as they inhibit *Giardia* attachment at a low concentration (5uM), but do not appear to effect host cell viability in our preliminary long term study, and importantly, also meet the requirements for Lipinski’s rule of 5, which suggest that these compounds have sufficient drug bioavailability [240].
3.5 Figures and Tables

IEC-6 High-Throughput Viability and Attachment Screen

Figure 1. IEC-6 High-Throughput Viability and Attachment Screen. An IEC-6 rat intestinal cell line was used to examine host cell viability and attachment in response to 240 NCI compounds previously shown to inhibit *Giardia lamblia* attachment. IEC-6 cells were distributed into 96-well plates and incubated in the presence of drug (50uM) for two hours. Plates were gently washed to remove detached cells. Remaining cells were stained for viability using EthD2 and then fixed and stained with syto-16 nucleic acid dye to visualize total cell attachment. Metamorph™ HTS software was used to collect automated images and cell counts. 4 images per well were taken (~20% surface area) using both GFP and DsRed filters, which resulted in 8 images per well. Cell counts were established by measuring the size and the intensity signal to noise ratio. Percent attached and viable were determined by comparing the number of attached and non-viable cell to that of untreated controls.
NCI Compounds Identified in IEC-6 High-Throughput Attachment and Viability Screen

<table>
<thead>
<tr>
<th>Drugs that *Weakly Affected IEC-6 Viability</th>
<th>Drugs that **Moderately Affected IEC-6 Viability</th>
<th>Total NCI Hits</th>
<th>Total Number of NCI Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>84 (~35%)</td>
<td>33 (~14%)</td>
<td>117 (~49%)</td>
<td>240</td>
</tr>
</tbody>
</table>

*Attachment and viability ≥80% for 2 independent trials
**Attachment ranged between 79%-50% and viability between 70%-59% for both trials

Table 1. NCI Compounds Identified in IEC-6 High-Throughput Attachment and Viability Screen.

To evaluate lead NCI compounds as potential drug candidates for the treatment of giardiasis the attachment and viability of IEC-6 rat intestinal cells was measured in response to 240 NCI compounds previously shown to inhibit parasite attachment by ~≥50% at a similar concentration (50µM). IEC-6 screenings were done in duplicate and attachment and viability were categorized as either “*weakly” or “**moderately” affecting host cell attachment and viability. Overall, 84 were shown to *weakly affect IEC-6 cell attachment and viability and another 33 were shown to **moderately affect IEC-6 cell attachment and viability. In all, 117 NCI lead compound were identified as potential drug candidates that merit further evaluation. These compounds represent 49% of the 240 compounds screened and ~6% of the original NCI diversity set library.
Identification of NCI Lead Hits

NCI Compounds that Inhibit *Giardia* Attachment

<table>
<thead>
<tr>
<th>240 Drugs Screened</th>
<th>Drugs that <strong>strongly</strong> inhibit <em>Giardia</em> attachment</th>
<th>Drugs that <em>moderately</em> inhibit <em>Giardia</em> attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs that <strong>weakly</strong> affect IEC-6 viability</td>
<td>19 Drugs ~ 8% <em>Giardia</em> Hits ~ 1% NCI Diversity Set</td>
<td>56 Drugs ~ 23% <em>Giardia</em> Hits ~ 3% NCI Diversity Set</td>
</tr>
<tr>
<td>Drugs that <em>moderately</em> affect IEC-6 cell viability</td>
<td>10 Drugs ~ 4% Primary Hits ~ 0.5% NCI Diversity Set</td>
<td>21 Drugs ~ 9% Primary Hits ~ 1% NCI Diversity Set</td>
</tr>
</tbody>
</table>

Figure 2. Identification of NCI Lead Hits. Previous screening of the NCI diversity set small molecule library (1,978 compounds) identified ~240 compounds that inhibited *Giardia lamblia* attachment by ≥50%. IEC-6 host cell viability and attachment screening of these 240 compounds identified 106 lead hit compounds that inhibited parasite attachment in the previous screening without affecting host cell viability or attachment (***Giardia* attachment inhibited ≥80%, +*Giardia* attachment inhibited 50%-79%, **IEC-6 attachment & viability ≥80%, †IEC-6 attachment 79%-50% & viability 70-59%).
Figure 3. Top Seven NCI Lead Compounds. 117 NCI lead compounds identified as inhibiting *Giardia lamblia* attachment with minimal to moderate effects on IEC-6 host cell viability and attachment were further evaluated for their ability to inhibit parasite attachment at a range of concentrations (50uM-1uM). A total of seven compounds were found to significantly inhibit parasite attachment at concentrations ranging between 50uM-10uM (A-G). Of these top seven NCI lead hits, 5857 (A), 38738 (B), 47949 (D), 82892 (F), and 658142 (G) were shown to significantly inhibit *Giardia* attachment at 5uM (Adj p< 0.05). All 7 NCI lead compounds showed characteristic dose-dependent effects on parasite attachment (H). Percent attachment represents the mean value of 5-9 values and error bars represent SEM.
Figure 4. *Giardia* and IEC-6 Cell Attachment and Viability. Pooled data from both the IEC-6 and *Giardia* attachment screens show that for the top 7 NCI drug candidates IEC-attachment and viability are not significantly affected, while *Giardia* attachment is significantly inhibited at the same concentration (50μM). Data represent the median percent attachment of 5-9 trials. Statistical analysis was done on actual cell numbers for both *Giardia* attachment (Adj P< 0.0001) and IEC-6 cell attachment and viability (Adj P >0.05).
Table 2. Characterization of Top Seven NCI Drug Candidates. The Developmental Therapeutics Program at the NCI/NIH provides a database (http://dtp.cancer.gov), which can be used to perform a basic chemical data search using the NSC identification number. Additional links allow for further searches, including searches for compounds with similar structures and/or similar compounds that follow Lipinski’s rule of 5, which evaluates the druglikeness of each compound.
CHAPTER 4

Future Directions:

Expression of Giardia and Human Actins

4.1 Introduction

The dynamic assembly and disassembly of actin microfilaments are required for many cellular functions, including cell structure and polarity, cellular division, intracellular transport, and cell motility and muscle contraction. The importance of the microfilament cytoskeleton makes it an appealing drug target against eukaryotic pathogens. However, this approach is complicated by the fact that actin is a well-conserved protein, and healthy non-pathogenic cells are also likely to be targeted, thus creating issues with toxicity. However, slight variations within the amino acid sequence of a protein can be significant enough to result in structural modifications. Although the proteins may function similarly, these modifications, particularly at binding sites, can be significant enough to either inhibit or promote the preferential binding of small molecule drugs [241] [242]. Recent studies have shed light on small structural differences in the actin microfilaments of parasitic protists (i.e. Leishmania and Toxoplasma), which affect
the binding of small molecule drugs [220] [243]. These results have evoked new interest in exploiting microfilaments as a potential drug target.

While actin is considered highly conserved among crown organisms, it is considerably less conserved than tubulin among the entire eukaryotic domain. *Giardia* actin, which was identified as a single copy gene by southern blot analysis, has a 58% amino acid identity to other eukaryotic actin sequences [221]. Moreover, a bioinformatics investigation of the *Giardia* cytoskeleton conducted in our laboratory concluded that the traditional eukaryotic microtubule protein machinery of *Giardia* is well-represented, whereas the traditional eukaryotic microfilament protein machinery was wholly absent beyond actin itself [181].

Moreover, current evidence has indicated that *Giardia* actin may also have modified structural differences, which could promote the differential binding of small molecule drugs. Primary evidence comes from sequence alignments and structural analysis that show an overall structural conservation between *Giardia* and mammalian actins, but slight localized differences within binding regions (Costanzi and Elmendorf, unpublished) (Table 1). This is supported by the fact that *Giardia* and human beta-actin are both 375 amino acids long, but only have an identity of 60.5% and a similarity of 72%. Further evidence comes from co-incubation experiments with *Giardia* and intestinal epithelial cells, in which fluorescent-labeled phalloidin failed to localized to *Giardia* actin, but showed
characteristic F-actin staining of the intestinal epithelial cells (Khoury and Elmendorf, unpublished). In support of this, drug treatment of *Giardia* parasites with the microfilament disruptor jasplakinolide, which shares the phalloidin binding site does not show characteristic cell detachment, as typically observed with other microfilament disrupting drugs (cytochalasins and latrunculins) [202] [209] (Khoury and Elmendorf, unpublished). This is consistent with homology modeling and *in silico* protein docking, which identified the shared phalloidin and jasplakinolide binding site of *Giardia* as having structurally significant differences and predicted that phalloidin would bind to *Giardia* actin with considerably poor affinity (Costanzi and Elmendorf, unpublished). Collectively, these findings implicate that *Giardia* actin has discrete structural differences that could influence the binding of small molecule drugs, and therefore presents itself as a meaningful target for the identification and development of new chemotherapeutics.

To further explore actin as a potential drug target we used a baculovirus expression system to express both *Giardia* actin and human beta-actin. The primary purpose for producing these proteins is to measure and compare the binding specificity and kinetics of small molecule drugs to both human and *Giardia* actin. To do this we will perform a high-throughput Biacore™ screen, which uses plasmon resonance spectroscopy to measure and compare the binding affinities and kinetics of the small molecule drugs in the NCI Diversity Set Library II. While
Diversity set II is not identical to Diversity Set I, it is largely overlapping (Diversity Set I is no longer available). The Biacore™ screenings will allow us to quantifiably measure the binding properties of small molecule drugs, and thus identify compounds that have a higher binding affinity to *Giardia* actin compared to human actin. Small molecule drugs that specifically bind *Giardia* are likely to interfere with actin-mediated processes, such as cell attachment, and thus would make good drug candidates.

Additionally, our homology modeling of *Giardia* actin will be used to perform high-throughput *in silico* molecular docking and pharmacophore searches to identify and predict compounds that will interact with *Giardia* actin. These compounds can then be added to our Biacore™ screen to evaluate their binding potential *in vitro*, and likewise the results of the Biacore™ screening will provide us with information to evaluate and refine our structural homology model and *in silico* screening protocol.

### 4.2 Materials and Methods

*Giardia* actin and human beta-actin were expressed in the baculovirus expression system, as instructed by the manufacture’s protocol (BD BaculoGold™ System, BD Bioscience, 560129). *Giardia* and human actin sequences were
amplified by PCR using Pfu DNA polymerase (Promega, M7741) and cloned into the pCR4-TOPO vector (Invitrogen, K458001). EcoRI digests were performed and actin inserts were sub-cloned into pAcHLT-A transfection vector, which includes an N-terminal His6 tag.

SF9 insect cells (BD bioscience, 21300L) were grown to ~90% confluency and recombinant baculoviruses were generated by co-transfection following manufacturer’s protocols. Plaque assays were performed to determine viral titer and amplification of viral stock was done at a low MOI (<1) to obtain a final viral titer of ~1.82 x 10^8 pfu/ml. For final protein expression, SF9 cells were infected with high MOI (~8) and harvested 3 days post-infection. Sonication was used to release protein from the insect cells.

Protein purification was performed per manufacture’s protocol using a Ni-NTA purification system under non-denaturing conditions (Invitrogen, K95001). Recombinant proteins were detected by SDS-PAGE and western blot analysis using anti-his antibody (Invitrogen, 460693). Dialysis was used to transfer recombinant proteins into G-actin buffer [243] using dialysis cassettes (3,500 MWCO) (Pierce, 66330). Dialysis was performed in 2L of G-actin buffer for 24 hours at 4°C with gentle stirring. Buffer was replace with 2L of fresh G-actin buffer and dialysis was continued for another 24 hours (total dialysis time was ~48 hours).
To perform Biacore™ screening, the recombinant His6 tagged actin proteins can either be covalently bound to Biacore™ sensorchips, or bound to Ni-NTA sensor chips via their His6 tag. Cytochalasin D, latrunculin A, and/or DNase I will be used as positive controls and for calibration purposes.

4.3 Results

To evaluate Giardia actin as a possible drug target, we have chosen to express recombinant his-tagged Giardia actin and human beta-actin in a baculovirus system for screening in a Biacore™ assay. Both actin proteins were individually cloned into pAcHLT-A transfection vectors, under the control of a poly-hedron late promoter. Both Giardia and human recombinant actins contain an N-terminal 6XHis tag, which will be used for purification purposes, as well detection.

Expressed recombinant proteins were released from the insect cells by sonication and the soluble recombinant proteins were separated and collected by centrifugation. Western blot analysis of supernatant from sonicated cells showed that ~50% of the recombinant protein was released from the pellet (Figure 1). Although overall protein yields from this system were less that expected, only small amounts of the proteins (picomolar) are required for Biacore™ screening.
Purification of the proteins was attempted using a Ni-NTA purification system that relies on the 6X-His tag. However, this step proved to be more difficult than anticipated. While proteins were consistently detected by SDS-PAGE and western blot analysis using anti-His antibodies (Figure 1), the recombinant proteins continuously failed to bind to the Ni-NTA resin, and thus were found almost exclusively in the flow-through following loading of the column. Although it is not entirely clear why either the human or the Giardia recombinant actin proteins were unable to bind to the Ni-NTA resin, it is likely that the 6X-His tag is folded within the proteins, and thus is not available for binding. This would explain why we were able to detect both human and Giardia actins on western blots following denaturing gel electrophoresis but were unable to purify using a Ni-NTA system.

Alternatively, recombinant actin may have polymerized during the process, potentially hiding the 6X-His tag. To test this theory, recombinant proteins were dialyzed into non-polymerizing G-actin buffer for 48 hours [243]. However, Ni-NTA purification and western blot analysis show that recombinant proteins were still unable to bind to Ni-NTA resin. These results led us to believe that the 6X-His tag was likely folded within the actin proteins’ tertiary structures.
4.4 Discussion

While we have successfully produced recombinant *Giardia* and human actins from a baculovirus system in insect cells, purifying the proteins has been problematic. Nonetheless, once we have obtained purified quantities of the recombinant proteins, we plan to immobilize the proteins (either by covalent binding or via the 6X-His tag) onto Biacore™ sensor chips. The recombinant actin proteins will then be used to screen the NCI Diversity Set II library for compounds that have a high affinity for *Giardia* actin. Given the wide spectrum of biological processes that actin is involved with, we expect compounds that bind to *Giardia* actin to significantly impair parasite functions, including – but not limited to – attachment. Importantly, we also expect compounds that preferentially bind to *Giardia* actin compared to human actin to have significant affects on parasites, without affecting IEC-6 host cells. These results can be tested and validated using our high-throughput attachment and viability assay previously developed for both *Giardia* and IEC-6 cells (see chapter 2 and chapter 3). Also, while not useful as new lead drugs, compounds that are identified as binding with equivalent affinities to both human and *Giardia* actin are still valuable as they can be used to investigate the mode of parasite attachment (see chapter 2).
In addition to screening the NCI diversity set II, we also intend to screen compounds identified in our *in silico* molecular docking and pharmacophore searches, performed in collaboration with Stefano Costanzi’s laboratory at the NIH. These screening approaches are designed to create a feedback loop in which non-conserved residues in *Giardia* actin that are predicted by *in silico* modeling to be important for binding specific ligands, can then be reproduced in a mutated recombinant actin, which can then be purified and evaluated in a comparative binding assay using the Biacore™ screen. Likewise, compounds that are identified as binding to *Giardia* recombinant actin in our Biacore™ screen can be used to perform structural *in silico* searches for compounds that are predicted to have similar binding properties, and to refine our structural homology model of *Giardia* actin. Give the nature of the feedback loop we intend to not only identify potential new drug candidates, but we also intend to identify compounds that will serve as tools for investigating the mode of parasite attachment. Information that is gained from our *in silico* molecular docking will provide us with information regarding the interaction of ligand with *Giardia* actin.
Expression of *Giardia* and Human Actins

Figure 1. Expression of *Giardia* and Human Actins. Expressed recombinant proteins were released from SF9 insect cells by sonication and the soluble recombinant proteins were separated and collected by centrifugation. Western blot with anti-His antibody shows production of recombinant *Giardia* and human actins. Actin proteins were expressed in baculavirus expression system (BD Bioscience, 560129).
**Amino Acid Differences Between Giardia and Human β-Actin in the Cytochalasin and Latrunculin Binding Pockets**

<table>
<thead>
<tr>
<th>Giardia Actin</th>
<th>Human β-Actin</th>
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<tbody>
<tr>
<td><strong>Cytochalasin Binding Pocket</strong></td>
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<tr>
<td>LEU59</td>
<td>GLN59</td>
</tr>
<tr>
<td>PHE206</td>
<td>ARG206</td>
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<tr>
<td>THR33</td>
<td>SER33</td>
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<tr>
<td>LYS183</td>
<td>ARG183</td>
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<td>GLU184</td>
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<td>LYS77</td>
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<td>THR303</td>
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<tr>
<td>PHE306</td>
<td>TYR306</td>
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<tr>
<td><strong>Latrunculin Binding Pocket</strong></td>
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<tr>
<td>MET375</td>
<td>PHE375</td>
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<td>VAL175</td>
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<td>VAL165</td>
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Table 1. Amino Acid Differences Between *Giardia* and Human β-Actin in the Cytochalasin and Latrunculin Binding Pockets. Although sequence alignments and structural analysis that show an overall structural conservation between *Giardia* and mammalian actins, slight localized differences within important binding regions have been detected (Costanzi and Elmendorf, unpublished).
Chapter 5

Drug Discovery in *Giardia lamblia*

5.1 Summary and Discussion

*Giardia lamblia* is one of the most prevalent intestinal parasites worldwide and is a significant contributor to diarrheal diseases [3]. Globally, diarrheal diseases cause nearly 4% of all deaths, and aside from respiratory illness, are the leading cause of death among children under 5 years [224]. While *Giardia* infections are frequently self-limiting and many individuals remain asymptomatic, chronic infections can persist [2], particularly in developing regions where poor sanitation is widespread and clean drinking water is often unavailable. Chronic infections are a particular concern in children, as they can result in long-term growth retardation and impaired cognitive development [235] [236]. Additionally, even asymptomatic individuals can pass high numbers of cysts, thus perpetuating the disease within populations. Consequently, *Giardia* has been added to the WHO Neglected Disease Initiative, which targets diseases that impair social-economic and developmental growth, and thus promote poverty [21].

Control of *Giardia* infections has proven to be difficult: worldwide sanitation is wholly insufficient to provide most of the population with clean
drinking water, it is a zoonotic disease with abundant wildlife, agricultural, and pet animal reservoirs, there is no vaccine, and the arsenal of chemotherapeutics is limited. Challenges with current drug treatments include, long treatment durations with high recurrence rates, and high incidence and severity of side effects for drugs with shorter durations of treatment. There have also been reports of drug resistance and contraindications, particularly for AIDS patients and pregnant women. And while there has been a slow trickle of new drugs brought into use against *Giardia* infections, metronidazole, the leading drug of choice, has never actually received an FDA indication for treatment of giardiasis [112] [106] [105]. Most importantly, the drugs currently in use are the result of fortuitous discoveries or secondary applications from other infectious disease treatment regimes. Fundamentally, there has never been a devoted drug development program for giardiasis.

A critical step in initiating and maintain an infection, is the ability of trophozoites to attach themselves to the intestinal epithelial lining of the small intestine. Failure to do so would result in clearance by peristalsis and ultimately a reduction in infection, as well as transmission [22]. This makes the process of parasite attachment an appealing drug target. Unfortunately, our knowledge regarding the mechanism of attachment is limited, and thus, greatly hinders our ability to perform targeted drug development that would specifically target
attachment as a virulence process. The lack of knowledge in the field regarding *Giardia* attachment is in part due to a deficiency of efficient and reliable tools.

With advancements made in high-throughput technology, small molecule inhibitor screens have become highly effective in identifying novel compounds that target specific cellular processes – simultaneously advancing efforts in both drug development and mechanistic understanding. Typically drug studies focus on one of two strategies, identifying ligand inhibitors of a known molecular target (bottom-up approach), or alternatively, identifying ligand inhibitors of a process or phenotype (i.e. parasite attachment) in the absence of any knowledge regarding the molecular machinery (top-down approach). Importantly, the top-down approach also allows researchers to use the small molecule compounds identified in the screens as tools to learn more about molecular machinery.

Here, we have devised a top-down phenotypic high-throughput screen to identify compounds that inhibit *Giardia* attachment, without affecting host cell viability. We have chosen to screen 1,978 compounds, which make up the NCI Diversity Set I small molecule library. This compound library is part of an “open repository” that consists of ~140,000 compounds. These compounds were narrowed down to ~72,000 based on availability, and then further streamlined to 1,978 by Chem-X, to define compounds that represented the greatest pharmacophore diversity (http://dtp.cancer.gov). Thus, in a relatively small
screen, an enormous diversity of chemical structures is evaluated. In turn, further analyses that pursue the larger family of structurally-related compounds can then extend the optimization of the inhibitors and probes.

The significance of our top-down approach is two-fold; (1) identifying compounds that will broaden our understanding of the process of attachment – and ultimately allow us to identify a larger range of potential drug targets which are currently not being investigated (see chapter 2) and (2) identifying compounds that inhibit parasite attachment may be useful in identifying and developing novel drug candidates for the treatment of giardiasis (see chapter 3).

5.2 Conclusions

5.2.1 Summary of Results

We have developed a high-throughput Giardia attachment assay to screen 1,978 compounds included in the NCI Diversity Set I small molecule library (The NCI/DTP Open Chemical Repository, http://dtp.cancer.gov). Compounds that inhibited parasite attachment by ≥50% (chapter 2) were further evaluated for their affect on IEC-6 host cell attachment and viability (chapter 3). Compounds that were identified as having a weak or moderate effect on IEC-6 host cells were
further evaluated at a wide range of concentrations (chapter 3). In all, we identified seven top lead drug candidates (NSC5857, NSC38737, NSC44480, NSC47949, NSC76026, NSC82892, and NSC658142) that significantly inhibit parasite attachment between 5-10uM, without affecting IEC-6 host cells at a much higher concentration (50uM). These compounds are currently under review for U.S. patent rights (U.S. Provisional Application No. 61/266,660, Georgetown Ref. No.: 2009-016). These compounds provide us with a strong foundation to move forward toward our dual goals of mechanistic discovery and drug development.

5.2.2 Exploring the Mechanism of Attachment

To further evaluate these compounds from a mechanistic perspective, we are conducting on-going qualitative and quantitative studies to advance both the mechanistic analysis and drug development goals. One component of this work is observation of parasite behavior and morphology following drug treatment to gain insight into the cellular pathways affected by the drugs. The drug effects can be categorized into 3 categories: compounds that disrupted parasite morphology, ranging from moderate to severe/lethal (3 compounds); compounds that did not affect gross parasite morphology but that caused flagellar paralysis (1 compound); and most interestingly, compounds that did not affect gross parasite morphology or activity but that prevented attachment (3 compounds). These findings therefore
narrow our efforts in future mechanistic studies to 3-4 compounds (NSC38737, NSC44480, NSC47949, and NSC76026) that appear to specifically inhibit parasite attachment, and from a mechanistic perspective are promising lead hits for target identification. To further this work, we have established collaborations with Dr. Christian Wolf’s laboratory to synthesize and biotinylate the three compounds for further use in pull-down assays and protein target identification.

5.2.3  *Advancing Drug Development*

From the drug development perspective, our seven potential candidates need to be further evaluated for their effect on long-term host cell viability, drug reversibility, drug solubility, and drug accessibility prior to the initiation of trials in a rodent model of infection. We have begun work on these new research goals.

While we have identified seven novel drug candidates, it is not likely they will all go forward into development. Foremost, more in-depth toxicity screens, including long-term viability assays need to be completed. We are currently in the process of doing this, and thus far preliminary evidence, based on qualitative observation (Helou and Elmendorf, unpublished) has suggest that three (NSC44480, NSC5857, NSC82892) of our seven candidates may have toxic effects on host IEC-6 cells at 15μM during a long-term incubation (2, 6 and 24 hours).
However, we did not see the same effect at 5uM, in which only NSC82892 appeared to cause cell death, nor did we observe cell death for any of the drugs at 1uM. So while NSC5857 appeared to induce host cell death at 15uM in our preliminary long-term study, it did not appear to cause host cell death for the same long-term duration at concentration of 5uM. Notably, NSC5857 was also found to significantly inhibit *Giardia* attachment at 5uM, and therefore remains a compound of interest.

Other considerations include Lipinski’s rule of five (Table 2). Importantly, three of our seven compounds meet these requirements, including, NSC38738, NSC658142, and NSC76026. Of these three compounds both NSC38738 and NSC658142 were found to significantly inhibit parasite attachment at 5uM, but yet didn’t appear to affect host cell viability in our long-term study, even at 15uM. While there is still much to do to evaluate these drug candidates, particularly with toxicity, our current most promising drug candidates for the treatment of giardiasis are, NSC38738 and NSC658142, as they inhibit *Giardia* attachment at a low concentration (5uM), but do not appear to effect host cell viability in our preliminary long term study, and importantly, they also meet the requirements for Lipinski’s rule of 5, which suggest it has sufficient drug bioavailability [240].
5.2.4  *A Closer Look at Actin*

My doctoral research also included a bottom-up approach that focused on the protein actin; again the goals of this work were two-fold: drug development and mechanistic exploration. The basis for this work comes from findings that drugs targeting the cytoskeleton can effectively inhibit parasite attachment. While there has been some disagreement in the literature, more recent studies have implicated microfilaments as having the more prominent role in this process. Unfortunately, the absence of many actin-related homologues has impeded the discovery or development of drugs that would specifically target the microfilament cytoskeleton [181].

Differences between the host and pathogen cytoskeleton have led to the development of drugs that target pathogen cytoskeleton function with minimal impact on normal host cell function: e.g. dinitroanilines selectively disrupt microtubules of protists and plants, but not fungi or animals, which is due to the differences in alpha-tubulin sequence and structure. Microfilaments have more recently emerged as a promising target for drug development for cancer and other diseases [244] [245] [246]. There is new evidence that differences in microfilaments of parasitic protists (e.g. Leishmania [220] [247] and *Toxoplasma*...
(243)) affect the binding of small molecules. These data indicate the promise of parasite actins as prospective therapeutic targets.

Therefore, in a bottom-up approach, I exploited *Giardia* actin as a potential drug target. Sequence alignment and structural analysis reveal an overall structural conservation between *Giardia* and mammalian actins with important localized differences in the inhibitor binding pockets. This is not surprising given that *Giardia* actin and human beta-actin both contain 375 amino acids, but only show 60.5% identity and 79.2% similarity. Importantly, structural homology modeling has identified key differences in known drug-binding pockets between human and *Giardia* actins, further supporting the potential to develop chemotherapeutics against this target (Stefano Costanzi, personal communication). Additionally, we also have functional evidence for differences in the phalloidin/jasplakinolide binding site. While initial studies based on structural homology modeling identified significant structural differences in *Giardia* actin, and further predicted phalloidin would bind relatively poorly to *Giardia* actin, we have been able to show that *Giardia* actin does not label with fluoresceinated phalloidin – despite staining of intestinal Mode-K cells (data not shown). Correspondingly, Jasplakinolide did not inhibit *Giardia* attachment– the only microfilament-targeting drug with no effect on parasite attachment (data not shown).
To accomplish this goal, I produced recombinant His-tagged *Giardia* and human actins in a baculovirus expression system with the goal of using these two proteins in a comparative drug-binding assay with Biacore™ technology. Unexpectedly, the production and purification of recombinant *Giardia* actin proved to be technically challenging for both our laboratory and a competitor’s laboratory (Zac Cande, personal communication). While we remain unsure of exactly why *Giardia* actin is problematic when expressed as a recombinant protein, I was successful in my efforts to eventually produce small, but sufficient quantities of the proteins (see chapter 4). Purification remains the next hurdle in this project – and the last one before a Biacore™ screen of the NCI Diversity Set II can be completed. My work here has advanced our efforts significantly, and thus represents an important complementary path of investigation in our collective work.


159. Li Zhi Yu, C.W.B., Rodney D Adam, *The two nuclei of Giardia each have complete copies of the genome and are partitioned equationally at cytokinesis.* Eukaryotic Cell, 2002. 1(2): p. 191-9.


206. Owen, R.L., P.C. Nemanic, and D.P. Stevens, *Ultrastructural observations on giardiasis in a murine model. I. Intestinal distribution, attachment, and


