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Identification Of Novel Antimalarials From Marine Natural Products For Lead Discovery

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IDENTIFICATION OF NOVEL ANTIMALARIALS FROM MARINE NATURAL
PRODUCTS FOR LEAD DISCOVERY

by

STEPHENIE M. ALVARADO
B.S. University of Central Florida, 2008

A thesis is submitted in partial fulfillment of the requirements
for the degree of Master of Science
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ABSTRACT

An estimated 500 million cases of malaria occur each year. The increasing prevalence of drug resistant strains of *Plasmodium* in most malaria endemic areas has significantly reduced the efficacy of current antimalarial drugs for prophylaxis and treatment of this disease. Therefore, discovery of new, inexpensive, and effective drugs are urgently needed to combat this disease. Marine biodiversity is an enormous source of novel chemical entities and has been barely investigated for antimalarial drug discovery. In an effort to discover novel therapeutics for malaria, we studied the antimalarial activities of a unique marine-derived peak fraction library provided by Harbor Branch Oceanographic Institute (HBOI). Within this unique library, we have screened 2,830 marine natural product (MNP) peak fractions through a medium throughput screening effort utilizing the SYBR Green-I fluorescence based assay, and have identified 253 fractions that exhibit antimalarial activity. From those inhibiting fractions we have identified twenty species of marine organisms that inhibit *Plasmodium falciparum* growth, from which thirty-five fractions were selected for further study. Among those thirty-five, eighty-three percent were also found to inhibit the chloroquine resistant strain of *P. falciparum*, Dd2. The most potent inhibitors were then screened for their cytotoxic properties using the MTT cell viability assay. Among the samples that exhibited potent inhibition of *P. falciparum* growth were fractions derived from a sponge of the genus *Spongosorites* sp.. This genus of sponge has been reported to contain the nortopsentin and topsentin class of bis-indole imidazole alkaloids. Nortopsentin A inhibited the parasite growth at the trophozoite stage with an IC_{50} value of 1.6 μM. This is the first report of antimalarial activity for this class of compound.

I would like to dedicate this work to my family and friends for their support, love, and encouragement. Thank you for all that you have done for me.

"Discovery consists of seeing what everybody else has seen and thinking what nobody has thought." – Albert Szent-Gyorgyi

"Don't be discouraged by a failure. It can be a positive experience. Failure is, in a sense, the highway to success, inasmuch as every discovery of what is false leads us to seek earnestly after what is true, and every fresh experience points out some form of error which we shall afterwards carefully avoid." – John Keats

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CHAPTER ONE: INTRODUCTION

Malaria is one of the most deadly parasitic diseases in the world. Over 41% of the world's population live in endemic areas, and according to the 2009 World Health Malaria Report, an estimated 500 million cases of infection and over 1 million deaths occur each year (WHO 2009). This number although alarmingly high, does not even include the large number of unreported cases that occur each year (Snow, Guerra et al. 2005). Susceptibility is not limited to a certain population of individuals, but the most severe cases of infection and mortality are seen in pregnant women and children under the age of five, due to the inability of these individuals to effectively fight off infection. Worldwide increases of malaria infection and mortality are associated with an increase in drug resistant parasites Many of these infections occur in sub-Saharan Africa where the incidence of drug resistant parasites is consistently rising, while the development of novel therapies has not (Eastman and Fidock 2009).

Pharmaceutical companies are perpetually looking to increase profits by discovering their next "blockbuster" drug. Research is most often times directed towards diseases that concern the consumers living within developed countries, and include areas such as cancer, cardiovascular diseases, and AIDS (Frearson, Wyatt et al. 2007). While these diseases result in a high mortality rate in developed countries, some of the world's most fatal diseases, mostly infectious diseases, are being neglected because of the poor economic state of the affected countries. Parasitic diseases such as leishmaniasis, trypanosomiasis, giardiasis, and malaria result in millions of deaths worldwide and have an enormous global impact, yet most of the drugs used to treat these diseases are over 30 years old and no longer effective (Renslo and McKerrow 2006). As of 2001, malaria infections were ranked the eighth highest contributor to the global Disability Adjusted Life Year (DALY) and second in highest contributor in Africa (Snow, Newton et al. 2003).

Parasitic diseases are prevalent in countries where patients do not have access to adequate health care, and are unable to pay for expensive therapies. This results in many patients waiting to seek therapy until the onset of severe symptoms (Frearson, Wyatt et al. 2007). There is an urgent need for inexpensive, effective, and safe drugs to combat these diseases, and within the past ten years there has been an increasing effort in parasitic drug discovery. This is mostly due to government agencies, like World Health Organization (WHO) and philanthropies, such as the Gates Foundation and Medicines for Malaria Venture (MMV). Yet, even with these organizations and the money that has been donated to identify new drugs and vaccines, we are still a long way from finding a cure, and better options for treatment.

Although malaria drug discovery research will primarily benefit developing nations, it is important to remember that even citizens of the developed world could be at potential risk. Because individuals of developed nations have more opportunities to travel for business and pleasure, the potential risk for infection among travelers could become a problem. Many of the military personnel are also at risk for acquiring malaria overseas while on duty. Even though the US number of infections and transmission rates are low, infection is still possible (Milhous and Kyle 1998; Snow, Guerra et al. 2005).

<u>Malaria Life Cycle</u>

An understanding of the parasite developmental cycle is critical to the design and development of novel therapies against infection. The majority of severe malaria cases that occur are due to infection by the parasite *Plasmodium falciparum*, which is the most deadly species of *Plasmodium* in humans. Four other species can also infect humans, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Although *P. knowlesi* was thought to be a simian malaria parasite, human infections have recently been reported in Thailand and Singapore (Jongwutiwes,

Putaporntip et al. 2004; Ng, Ooi et al. 2008).Overall, the life cycle stages of these species are

quite similar with the parasites having three distinct stages during their life cycle, two asexual

stages, and one sexual stage of development, Figure 1. Infection of a vertebrate host begins by

the injection of sporozoites, an asexual form of the parasite, into the host when an infected

female *Anopheles* mosquito takes her blood meal. If the parasites are able to overcome the

barriers mounted by the host's immune response to infection, they travel to the liver through the

blood stream and cross the sinusoidal layer and basement membrane to infect the hepatocytes

(Beier and Vanderberg 1998) . Once inside the hepatocytes, the parasite forms merosomes,

which are aggregates of merozoites. An exception is the infection caused by *P. vivax* and P.

ovale that are able to remain dormant in the liver as hypnozoites for extended periods of time.

When the merosomes rupture, they release thousands of merozoites that travel through the blood

stream to invade erythrocytes; this begins the asexual, developmental stages of the parasite

(Beier and Vanderberg 1998).

   During the developmental stages of *Plasmodium*, the parasite undergoes multiple DNA

replication cycles. In the case of *P. falciparum*, this occurs once every 48 hours. The parasite

first invades the erythrocyte as a merozoite, where it forms a ring-like structure, named so

because of the shape it portrays on a Giemsa stain. At this stage, the parasite begins to increase in

size, creating a suitable environment for its growth and survival within the erythrocyte (Ayong,

Pagnotti et al. 2007). DNA replication and synthesis then begins in the proceeding trophozoite

stage. The transition from the trophozoite form to schizont stage is difficult to pinpoint, because

the increase of DNA content continues throughout the schizont stage (Grimberg, Jaworska et al.

2009). Later, the formation of multinucleated schizonts appears in the RBC. This multinucleated

form is often referred to as a segmented schizont, which then ruptures and releases merozoites

into the blood where new infections can take place. In order for the parasite to infect other hosts, it must be taken up by the mosquito and injected once again.

The formation of gametocytes is simultaneously occurring during the developmental cycle. In *P. falciparum*, the development of macrogametocytes and microgametocytes, female and male, respectively, form as a result of both environmental and innate triggers (Sinden 1998). The fertilized zygote is taken up during the blood meal of the female Anopheles mosquito. After a 12-24 hour period the development into ookinetes occurs, which is followed by invasion of the mosquito's mid-gut epithelium (Sinden 1998). Once the ookinete is present in the mid-gut basal lamina, it develops into the oocyst, which is surrounded by a capsule, and undergoes a series of nuclear divisions to form sporozoites. These sporozoites mature and subsequently invade the salivary glands of the mosquito, where they are ready for re-injection into a new host and re-initiating the parasite transmission cycle (Sinden 1998).
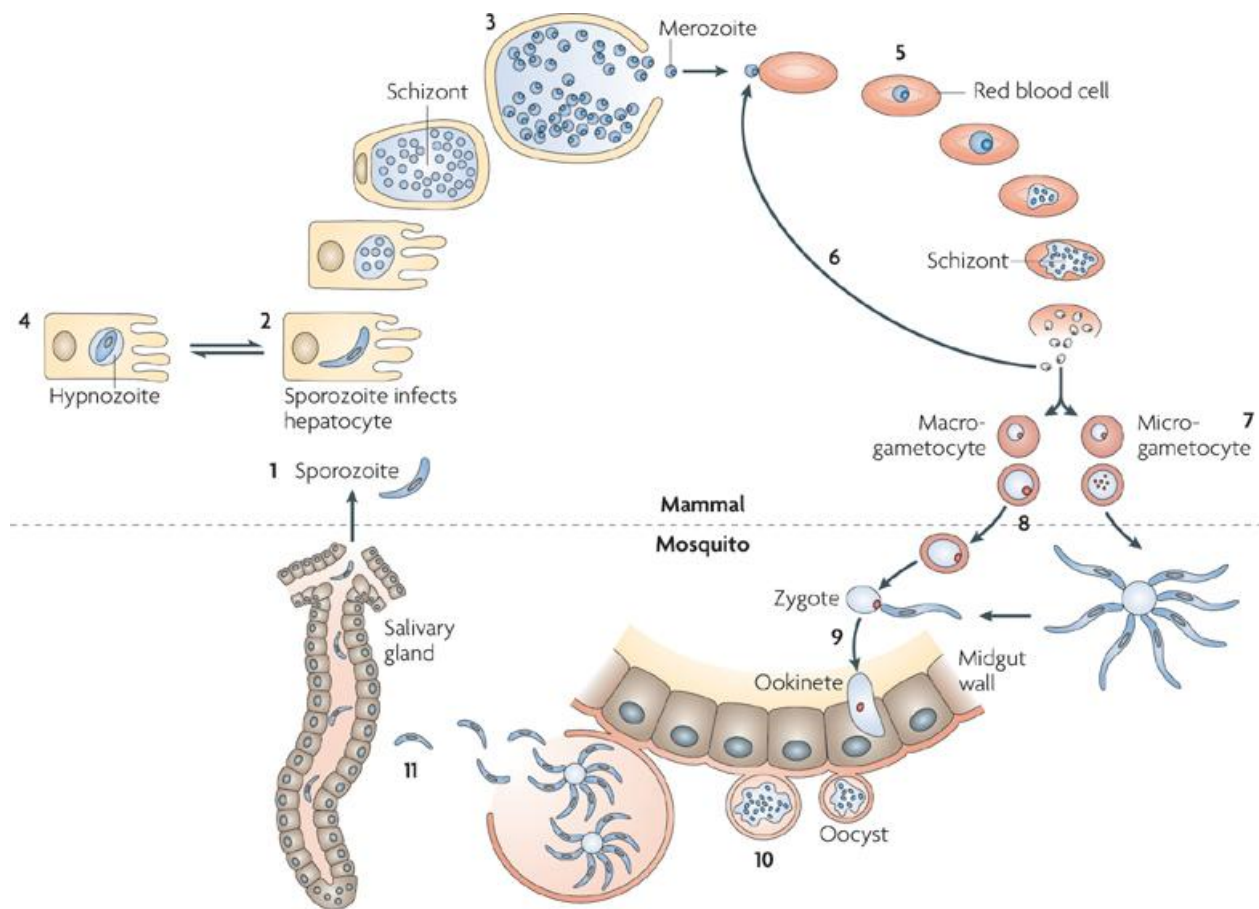
Figure 1 *Plasmodium* life cycle (Wells, Alonso et al. 2009).

Disease Prevention Approaches

During the 1950s and 1960s there was a movement to eradicate malaria through the Global Malaria Eradication program. This goal was carried out by administering chloroquine and pyrimethamine as well as spraying of DDT to eliminate the parasite vectors (Talisuna, Bloland et al. 2004). Since the end of the Global Malaria Eradication Program in the early 1970s, due to the increase in resistance and lack of structured health care systems, there has been a need to find more effective ways of treating and preventing *Plasmodium* infection (Wright, Fritz et al. 1972). An effective method currently used for prevention includes the use of special bed nets that contain pyrethroid insecticides. These bed nets are able to kill the Anopheles mosquito vectors that carry the parasite thus preventing transmission of infection. Resistance to these insecticides is a concern in many areas where bed nets are currently in use. Research efforts to find new and effective insecticides are ongoing (Greenwood, Fidock et al. 2008). It is apparent that although these nets serve as an excellent first line of defense against infection, development of effective preventative and/or therapeutic approaches are needed to control the spread of disease.

Development of an effective vaccine would be an ideal means for malaria prevention. The most promising vaccine candidate is currently in phase III clinical trials. Glaxo-Smith Kline's RTS,S vaccine targets the pre-erythrocytic stage of *P. falciparum* (GlaxoSmithKline 2010). During this stage, there are many barriers, like the activation of cytotoxic lymphocytes, which the parasite must overcome in order to efficiently invade hepatocytes. The limited amount of sporozoites present within the human host during this stage also makes it an attractive vaccine target (Greenwood, Fidock et al. 2008). Live attenuated vaccines were created in the 1950s and 1960s that were lacking essential liver stage proteins, 6-cysteine proteins and parasitophorous vacuole (PV) deficient protein. Both of these proteins are required for the formation of the PV,

an environment that shields the parasite from the host immune response. Symptoms associated with uncomplicated malaria infection, which include fever, nausea, headaches, and chills, occur during the developmental stage of the parasite, for which natural immunity is acquired throughout an individual's lifetime (Greenwood, Fidock et al. 2008; Pierce and Miller 2009). Mimicking the naturally acquired immunity can potentially result in a vaccine that prevents the onset of severe disease in younger patients (Pierce and Miller 2009). Other promising vaccine candidates include those that protect against placental malaria which affects women who are most often having their first or second child (Pierce and Miller 2009). Studies on antibodies which block the adhesion of *P. falciparum* parasites to the placental receptor, chodroitin sulfate A, have been shown to result in higher birth weights and longer gestational periods, and could result in an effective vaccination during pregnancy (Duffy and Fried 2003). Identification of an effective vaccine would result in a decrease in the transmission rate of infection, and reduce the need for novel therapies. But the success for malaria vaccine discovery has been limited, and until a vaccine can be safely administered to all individuals within affected regions, there will continue to be a growing need to discover and understand novel antimalarials.

<u>Available Malaria Therapeutics</u>

Quinolines

One of the most potent antimalarials to date is quinine, which has been used in many forms since the earliest reports of malaria in the world. Quinine is a naturally occurring chemical compound found in the bark of cinchona trees, and throughout its use, it was identified as a chemotherapeutic for the treatment of malaria (Meshnick 1998; Desowitz 1999). Quinine is not preventative, and was only used once the symptoms of disease began to occur. Since its discovery, several derivatives of the compound have been developed with increased potency

against malaria parasites. One of the first compounds synthesized was chloroquine, a 4-aminoquinoline, but due to inaccurate reports of toxicity, the drug was not used until the mid 1940s. During this time, the United States Military studied its structure and re-examined its toxicity, ultimately leading to the determination that it was a safe drug for prophylactic and chemotherapeutic use (Desowitz 1999).

For the past 50 years, chloroquine has been the gold standard for treatment of malaria infection for all four *Plasmodium* species. The use of chloroquine over many other chemotherapeutics in the past has been attributed to its effectiveness, as well as the safety of the drug for pregnant women and children (Nwaka and Hudson 2006). Because of its widespread use, the efficacy of the drug began to decline with the emergence of chloroquine drug-resistant strains. Reports of resistance first appeared in 1957 along the Thai-Cambodia border and then spread throughout Thailand by 1960. Another, foci of resistance emerged in 1960 in Colombia, while African countries began to see resistance in the mid-1970s (Talisuna, Bloland et al. 2004). Since then, chloroquine resistant strains of *P. falciparum* have been documented in every country that is affected by malaria infections (Fidock, Rosenthal et al. 2004).

Chloroquine's mechanism of action is still being studied for a more in depth understanding of the parasite biology, and although some debate over the topic remains, it is apparent that chloroquine is involved in disrupting the processes of the digestive vacuole. The digestive vacuole utilizes aspartic and cysteine proteases to degrade hemoglobin and obtain amino acids for protein synthesis. This process is the most active during the late ring and trophozoite stage of the parasite, and it has been suggested that chloroquine acts by inhibiting these activities (Gluzman, Francis et al. 1994; Francis, Sullivan et al. 1997; Krogstad and Dibyendu 1998).Chloroquine has been hypothesized to act on the food vacuole in a few different

ways. During the degradation of hemoglobin, the free heme that accumulates, as a result, is membrane toxic to parasite and the RBC. In order to overcome this, the parasite binds the heme into a non-toxic form called hemozoin. Chloroquine is thought to bind to free heme present after hemoglobin degradation. This heme is then no longer able to be bound into a non-toxic form, and kills the parasite. Understanding the mechanism of action for a drug is most often thought to be a starting point for also understanding its mechanism of resistance. In the case of chloroquine, the factors related to both mechanisms are associated with the digestive vacuole, but how they act on the parasite are unrelated, and have required researchers to develop an understanding of resistant parasites.

Chloroquine resistance is a major problem within endemic countries, and there have been many efforts to reduce and reverse the occurrence of resistant strains of *P. falciparum*. The mechanism of chloroquine resistance within the parasite has been shown to occur due to multiple mutations within the chloroquine resistant gene, *Pfcrt*. This gene is responsible for producing the PfCRT transporter that localizes to the digestive vacuole and causes an efflux of chloroquine out of the digestive vacuole where the drug acts (Wellems and Plowe 2001). Verapamil is a calcium channel blocker that is sometimes prescribed for patients with irregular heartbeats and high blood pressure. It works by relaxing blood vessels in order to allow the blood to flow more easily to the heart (MedlinePlus 2010). It has been shown that verapamil increases the accumulation of chloroquine within resistant parasites. The increased accumulation was only seen in resistant strains of the *Plasmodium*, and has therefore led researchers to hypothesize that the occurrence of resistant parasites is related to calcium transport. Another suggested mechanism of resistance is a change in pH within the digestive vacuole. Chloroquine is a weak base and is localized to

this acidic compartment where it presumably inhibits the ability of the drug to effectively kill the parasite (Reeves, Liebelt et al. 2006).

In order to overcome the problem of chloroquine resistance, a series of chloroquine analogs were synthesized. One of the first attempts aimed at synthesizing new drugs was the combination of two quinoline structures attached by a linker. The side chains of this structure were modified in order to create a series of related chemical compounds. One of the more promising chemical entities was mefloquine, which was introduced as an antimalarial in 1975 (Raynes 1999). Mefloquine's mechanism of action appears to be similar to that of chloroquine, in that it interferes with the food vacuole's degradation of hemoglobin. Although mefloquine was initially active against chloroquine resistant parasites, an increase in mefloquine resistant parasites has occurred since its introduction. Primaquine is another quinoline based drug, 8-aminoquinoline that was synthesized as a second generation drug and was used to combat drug resistant *P. falciparum* parasites. It has also been found that it effectively prevents relapse infections from occurring in patients infected with *P. vivax* by killing dormant hypnozoite liver stage parasites (Wells, Alonso et al. 2009). Quinine and its derivatives were at one time effective at treating *Plasmodium* infection, but due to the emergence of resistant parasites this form of treatment is no longer effective at clearing infection. The need to identify novel chemical structures and compounds that are potent against *P. falciparum*, as well as targeting different cellular targets, has led to the development of other well known antimalarials.

Antifolates

Antifolates are agents that inhibit folate synthesis in the malaria parasite. The folate synthetic pathway in *P. falciparum* has been suggested to be mediated by a *de novo* pathway in which the parasite synthesizes folate for the reduction of GTP into tetrahydrofolate (Ferone

1977); however as a salvage pathway for folate synthesis has also been identified, it is most likely not the main source of folate (Gregson and Plowe 2005). Tetrahydrofolate is necessary for the metabolism of amino acids, nucleic acids, and L-glutamate for the parasite's continuation through its cell cycle (Cowman 1998). Inhibitors of this pathway eventually halt DNA synthesis and kill the parasite.

There are many enzymes involved in the folate synthetic pathway (Figure 2), and there are two that are the targets for antifolate drugs used as chemotherapies for the treatment of malaria. The first enzyme within the pathway, which has been shown to be inhibited by Sulfadoxine is dihydropteroate synthase (DHPS). This enzyme works by inhibiting the enzymatic activity of DHPS to condense p-aminobenzoic acid (PABA) along with 6-hydroxymethyl-7,8-hihydropterin pyrophosphate to yield 7,8-dihydropteroate substrate for subsequent dihydrofolate synthesis. Sulfadoxine is structurally similar to PABA and interferes with the condensation reactions, thus halting the folate synthesis pathway and killing the parasite (Fidock, Su et al. 1999).

Other antifolates have also been identified for their therapeutic and prophylactic properties against malaria. These include marketed antimalarials which inhibit the enzyme dihydrofolate reductase (DHFR), like pyrimethamine, cycloguanil and their derivatives. Both pyrimethamine and cycloguanil are structurally similar to folate and inhibit the ability of DHFR to reduce dihydrofolate to tetrafolate (Fidock, Su et al. 1999; Gregson and Plowe 2005). Clinically, Sulfadoxine is used in combination with pyrimethamine to prevent infraction as well as treat patients who have acquired infection by chloroquine resistant parasites. The combination of these drugs is marketed as Fansidar, and is relatively inexpensive, or at least comparable in price to chloroquine (Cowman 1998).

Since the 1940s, antifolates have been available to treat malaria, but due to the high reports of drug resistance and the slower rate of parasite death, chloroquine remained a more attractive choice for therapy. One of the major problems with antifolates is that when used as a mono-therapy, there is a rapid development of resistant *P. falciparum* strains. But, a study in 1959 found that when pyrimethamine and sulfadoxine were combined, the combination was more effective than chloroquine alone; however. resistant parasites were found to develop after long periods of treatment (Gregson and Plowe 2005). This resistant phenotype has been analyzed using laboratory strains of the parasites, and it has been shown that DHFR inhibitors are no longer effective when a series of point mutations at positions 16, 50, 51, 59, 108, and 164 in the *Pfdhfr* gene occur (Gregson and Plowe 2005). These mutations occur within the active site of the enzyme's catalytic pocket. Mutations in the gene, coding for the DHPS enzyme also occurs in drug resistant parasites. One of the explanations for the occurrence of these mutations is that sulfadoxine and pyrimethamine are not rapidly cleared from the body. This allows for the selection of resistant parasites over time. Modifications to both sulfadoxine and proguanil have yielded a new class of antifolates, chloroproguanil and dapsone that are cleared more rapidly from the body, and were once a considered to be a promising therapy which would have a rate of resistance development (Hyde, Dittrich et al. 2008). During phase III clinical trials of the drug combination, it was shown that there was a significant amount of hemoglobin degradation that occurred in patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency. Because ten to twenty-five percent of the sub-Saharan population possessing this deficiency, this new class of antimalarials would have done more harm than good (GlaxoSmithKline 2008). Other antimalarials have also been developed and offer potency as well as a lower risk of negative side effects.
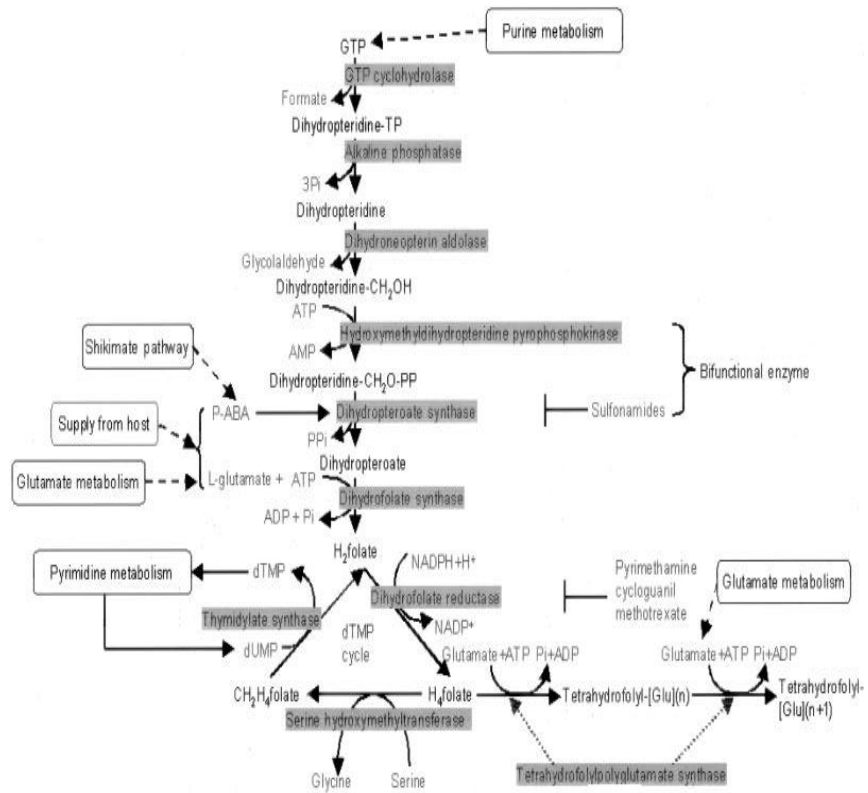
Figure 2 Folate biosynthetic pathway (Gregson and Plowe 2005).

Artemisinin

Artemisinin, also known as qinhao, is a natural product derived from the Chinese herbal medicine from *Artemesia annua*, and has been found to be a potent antimalarial against chloroquine resistant and antifolate resistant drugs (Meshnick 1998). While discovery of artemisinin was a major advancement in the search for novel antimalarials, there are some problems associated with the use of artemisinin and its derivatives as a first line of defense against *P. falciparum* infections. The first is in the method of production for artemesinin. In order to make large quantities of artemesinin, it must first be extracted from the plant and then modified into its derivative forms, dihydroartemisinin, artemether, arteether, and artesunate (Eastman and Fidock 2009). The length of time and labor required to produce the semisynthetic derivatives makes the cost of artemisinin more expensive than the other antimalarials currently being used (Lubell, Yeung et al. 2009). The recent demonstration that the precursor for the production of artemisinin could be produced in genetically engineered yeast, *Saccharomyces cerevisiae* generates the hope that issues related to the availability of artemisinin can be overcome through biotechnology (Ro, Paradise et al. 2006).

Although progress has been made to reduce the production cost of artemisinin based drugs, there have been concerns about its toxicity. Artemisinin has been shown to cause neurotoxicity and embryo lethality in animal models (Gregson and Plowe 2005). Clinical studies that have been performed with children and pregnant women have not yielded similar results (Eastman and Fidock 2009). The concerns for the possible side effects of artemisinin indicate a need for a more thorough understanding of how the drug targets the *Plasmodium* parasite.

Although the exact mechanism of action of artemisinin is not known, recent discoveries show that artemisinin locates to the digestive vacuole and may somehow affect heme and iron

within the parasite as well as potentially causing an increase in free radicals (Meshnick 1998; Eastman and Fidock 2009). In order to further determine the mechanism of action, studies have successfully shown that the peroxide bridge within the heme iron is reduced within the digestive vacuole. The generation of free radicals in the presence of artemisinin has been thought to alkylate and oxidize lipids and other proteins within *P. falciparum*, and could potentially lead to parasite death (Eastman and Fidock 2009). This information is critical to the understanding of the parasite's death and the possible mechanism of resistance.

The rate at which the development of resistance occurs is a major concern as with any new antimalarial drug, and it is important to determine the mechanism by which this might occur. Because artemisinin has a short half life, and does not remain in the body for extended periods of time at low concentrations, resistance in clinical isolates is not as likely to occur as compared to other antimalarials with longer half lives. In order to better understand a potential resistance mechanism, parasites were placed under selective pressure to isolate resistant lines with mutations (Witkowski, Lelievre et al. 2010). Some studies have shown data indicating that mutations associated with the *pfatp6* and *pfmdr1* genes occur within parasites that are less sensitive to artemisinin. PfATP6 is a calcium dependent ATPase found within the endoplasmic reticulum, and PfMDR1 is a multidrug resistance protein 1(Eastman and Fidock 2009). One of the therapeutic approaches to circumvent the possible rise of resistant parasites has been the use of an artemisinin regime that is combined with other antimalarials. Over the past few years, artemisinin combination therapies (ACTs) have become the first line of treatment for patients with malaria.

ACTs are combinations of artemisinin derivatives, artenmol, artmether, arteether, artesunate, and DHA, with other known antimalarials, such as sulfadoxine and mefloquine,

whose structures are shown in figure 3. The efficacy of this combination is evident in the success

in treating patients without an increase in drug resistance. Although some reports have indicated

a potential decrease in sensitivity to artemisinin in patients who have undergone therapy, the

clinical isolates were tested in culture and are still susceptible to artemisinin treatment

(Witkowski, Lelievre et al. 2010). The success of ACTs is a major breakthrough for antimalarial

development, but evidence has shown that resistant parasites can arise, and with the cost

associated with artemisinin treatment as well as potential negative side effects, there is still a

need for novel, inexpensive, and safe antimalarials.
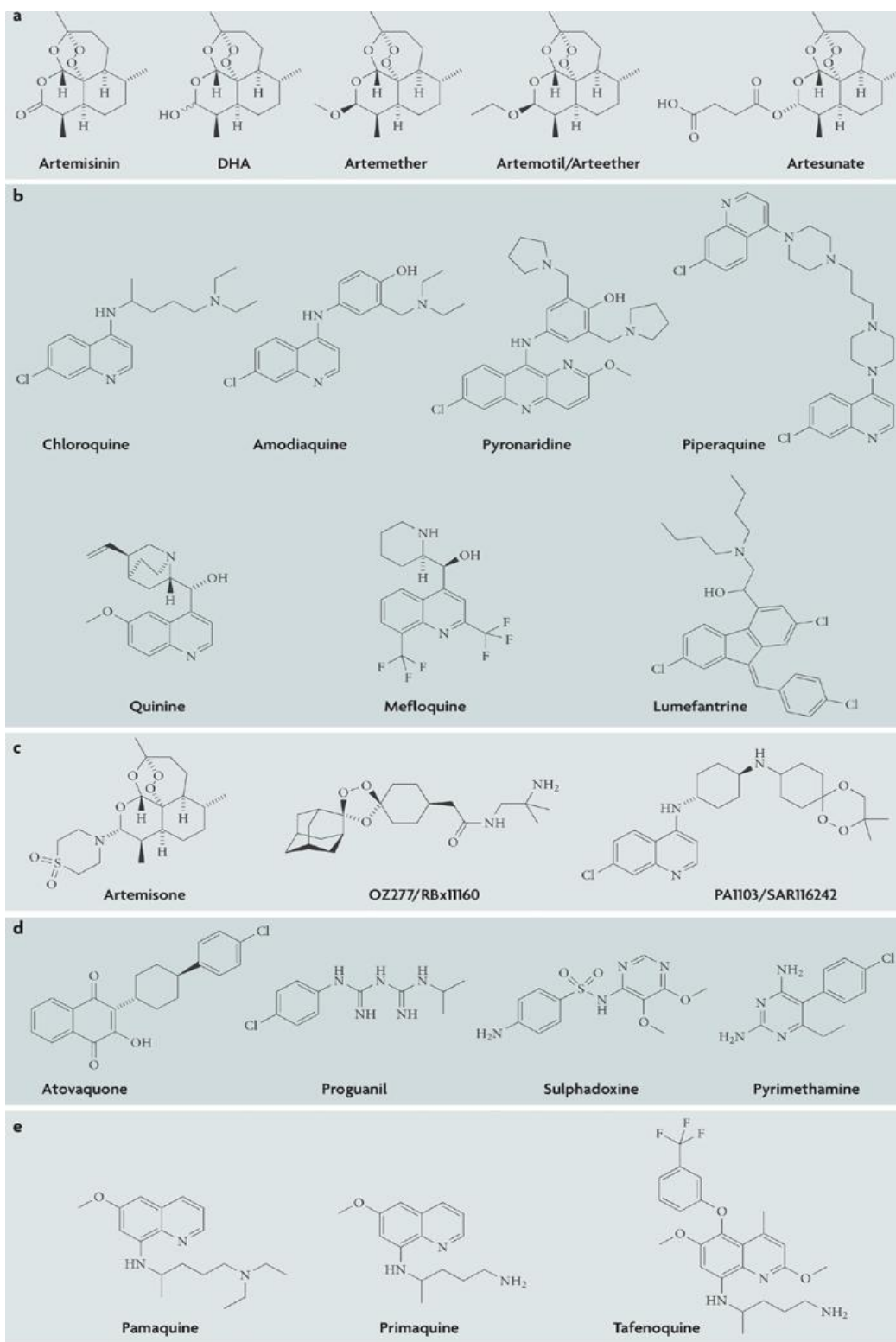
Figure 3 Antimalarial compound chemical structures (Wells, Alonso et al. 2009).

Antimalarial Drug Development

The past five years has seen an increased effort in antimalarial drug development due to an increase in the number of partnerships that have been formed between the pharmaceutical industry, academia, and government institutions. These partnerships are beneficial in that much of the discovery phase of research is performed in an academic setting, which reduces the cost of development for pharmaceutical companies. Once a compound, or series of compounds, is identified, the pharmaceutical company can become involved in the development of the compound into a potentially marketable drug. Through these key partnerships, the pipeline for new antimalarials has increased over time to the portfolio that now includes a number of new chemical scaffolds as well as improved analogs of previously identified antimalarials. The portfolio that has been outlined by MMV also includes antimalarials with novel targets as well (Olliaro and Wells 2009).

The MMV portfolio includes compounds that are at the beginning stages of lead optimization, to those that are undergoing phase III clinical trials. Of the twenty-five compounds that are currently being developed, there is only a handful with novel properties. Some of these include the lead optimization of an antimalarial that inhibits the parasite enzyme, dihydroorotate dehydrogenase, DHODH, which is required for the pyrimidine synthesis (Baldwin, Michnoff et al. 2005; Olliaro and Wells 2009). Novel molecular targets include the parasite's mitochondria, which is inhibited by the antimalarial 4-pyridone. 4-Pyridone is currently in phase I clinical trials and works as a mitochondrial electron transport inhibitor (Yeates, Batchelor et al. 2008; Olliaro and Wells 2009). The molecular targets that are currently being focused on for lead discovery and optimizations are summarized in figure 4 from the review by Wells, *et. al*. One of the more attractive targets for drug development includes the apicoplast, which is a unique organelle of

Apicomplexa intracellular parasites. The apicoplast is a four membrane bound plastid that is essential for *P. falciparum* survival in the human host as it is responsible for a number of essential biosynthetic pathways, and would be an ideal drug target because of its presence within the parasite alone (Kalanon and McFadden 2010). Along with novel molecular targets, the portfolio includes new combinations of antimalarials in fixed-dose formulas (Wells, Alonso et al. 2009). Although new targets and chemical scaffolds have been identified, there is still a lack of novelty within the portfolio. Therefore there is still an urgent need for antimalarials that work better than those that are currently prescribed, as well as those that cost less. In order to obtain these new drugs, there are several approaches that can be pursued.

The first is drug discovery through rational drug design. Using this approach, antimalarials are developed by first identifying a target and validating it as being essential for parasite survival. With the completion of the *Plasmodium falciparum* 23-megabase genome in 2002, rational drug design is now a viable option in antimalarial drug discovery (Gardner, Hall et al. 2002). Once a target has been validated, synthesis of small molecule inhibitors are developed and screened for their potency. Target validation is also important for piggy-back drug discovery, which focuses on using inhibitors of homologous targets in other diseases as a starting point for identifying *Plasmodium* inhibitors (Nwaka and Hudson 2006). But validation of molecular targets requires an in-depth knowledge of how the 5,000 genes identified in *P. falciparum* act on parasite development and survival. Many of the genes that were identified do not contain homologous sequences, making it difficult to identify these potential targets. With time, this dilemma will be addressed with continued study and understanding of the parasite, but not fast enough for the development of novel antimalarials within the next few years. In order to combat drug resistant strains at this time, other approaches need to be taken to identify novel antimalarials. De novo

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drug discovery offers the possibility of identifying novel antimalarials from chemical libraries that contain synthetic compounds as well as natural products . High throughput screening methods have been developed and validated, using whole parasite assays. This allows for a rapid and accurate account of parasite inhibition by these various chemical libraries which have been developed through the use of biological, biochemical, and structural rationale (Nwaka and Hudson 2006).

Molecular target	Function	Key objective of drug development	Development stage
Dihydrofolate reductase	Folate biosynthesis	Overcome existing resistance	Preclinical
Dihydroorotate dehydrogenase	Pyrimidine synthesis	Show selectivity and potency *in vivo*	Lead optimization
Purine nucleoside phosphorylase	Nucleoside synthesis	Provide clinical proof-of-concept	Phase II for autoimmune disease
Adenosine deaminase	Nucleoside synthesis	Show selectivity and potency *in vivo*	Preclinical
Cytochrome bc1 complex	Mitochondrial respiration	Overcome existing resistance and show selectivity for parasite target	Preclinical and Phase I
Subtilisin-like protease	Egress from erythrocytes	Lead discovery	Discovery
Falcipains	Proteolysis of haemoglobin	Show selectivity against host proteases and develop pharmacophores that do not cross-react with host thiols	Lead optimization
Fatty acid biosynthesis	Apicoplast lipid synthesis	Lead discovery	Discovery
Histone deacetylase	DNA replication	Show selectivity for parasite target	Discovery
Kinases	Signal transduction	Show selectivity for parasite target	Discovery

Figure 4 Molecular Targets for antimalarial drug discovery (Wells, Alonso et al. 2009).

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Marine Natural Products

In the last two decades, the majority of small molecule drugs that have been developed worldwide are analogs of compounds isolated from natural products. For example, studies on parasitic diseases have yielded ten natural product derived drugs out of the fourteen that were developed between 1981 and 2006 (Newman and Cragg 2007). Antimalarials are excellent examples of small molecule drugs derived from natural products exhibiting potent activities. For example, chloroquine and artemisinin have been used medicinally throughout history, and have been developed into marketable drugs. Due to the ease of accessibility, most of the focus of study for these naturally occurring products has been on terrestrial plants, with ninety-one compounds in clinical trials in 2007 (Li and Vederas 2009).

Terrestrial plants, as stated above, have successfully yielded many drugs, but marine biodiversity is relatively under studied. Oceans cover around seventy percent of the Earth's surface, and are an enormous source of biodiversity, with 34 out of the 36 phyla of life being represented (Gul and Hamann 2005). According to one report, the estimated number of invertebrate and algal species within the ocean is at least 200,000, but could include many more species that have yet to be studied (Wright 2000). In 2004, alone, there were reports of at least 716 new chemical entities that were discovered with 88 new compounds that were derived from marine natural products (Hill 2007). Although success with antiparasitic drugs from marine natural products has been limited, the opportunities for identifying novel antimalarials has sparked interest among researchers due to the success that has occurred within the area of anti-cancer therapeutics. One of the first successes was in the discovery of drug candidate from the Caribbean sponge, *Tethya crypta*; which is now used to treat leukemia and non-Hodgkin's lymphoma patients, while other anti-tumor candidates are currently in clinical trials (Wright

2000). In order to have success in marine natural product drug discovery, investigators must have an understanding of how the drug discovery process works as well as access to the equipment needed to acquire these products.

Harbor Branch Oceanographic Institute (HBOI), located in Ft. Pierce, Florida, has the skills and equipment needed to create a unique chemical library from marine natural products. What makes HBOI's collection of marine specimens unique is the means by which they collect their samples. HBOI is one of six institutions worldwide that have manned submersibles that are able to collect marine organisms from depths as far down as 915 meters. There are three submersibles that are equipped with arm attachments, like a scoop, claw hand, and suction adaptor that allow them to collect specimens from the mid-water and benthic zones. Using these vessels, HBOI has gained access to many areas within the ocean that have been unexplored for novel compounds, including deep and shallow reefs, grass beds, caves, pilings, and even shipwrecks, as well as other areas. Between its inception in 1984 and 2000, HBOI's effort to identify bioactive components has yielded a collection of 20,000 marine invertebrate and algal species (Figure 5). These include a majority of sponges, but represent a diverse selection of phylums found within the marine ecosystem. Once the specimens are collected, they are stored in ethanol and are processed and enriched at HBOI and other partnering academic and industrial institutions (Wright 2000).

Some of the successes that HBOI have had include specimens collected from sponges of the genus *Spongosorites*, which are most often found with gastropod molluscs. This sponge has been shown to contain many bioactive compounds, one of which, Topsentins, that has been studied for its anti-inflammatory properties. Topsentins are bis(indole) alkaloids and are planar in nature, which would allow for them to be synthesized at a relatively low cost, making the

product less expensive for consumers. Topsentin and bromotopsentin were first to be isolated, while similar compounds have been isolated by HBOI, and include Nortopsentin A-C, dragmacidin D, 2,2-bis(6'-bromo-indole-3'-yl)ethyl amine. Another compound that has shown promise for HBOI is Discodermilide, which was isolated from *Lithistid* sponges. The activity of this compound was first thought to act as a immune suppressant, but it has also been shown to have antitumor properties by acting on tubulin polymerization (Wright 2000). HBOI has also had success with other marine specimens as well, and is currently continuing their research to investigate other areas of therapeutics (Wright 2000).

Our laboratory partnered with HBOI to identify potential novel antimalarials from marine natural products. During this investigation our aim is to screen marine natural products from HBOI's peak fraction library. This library contains a diverse amount of crude extracts that exhibit inhibition on a panel of tumor cell lines by less than or equal to 40% at 5 μg/ml. The fractions within the peak library are also highly enriched and have been separated through the use of medium pressure liquid chromatography, reverse phase high pressure liquid chromatography along with UV index reading. According to the investigators at HBOI, nuisance compounds can be eliminated based on the observation that many small molecule drugs have a UV chromophore. The elimination of nuisance compounds allows us to avoid the screening of inactive fractions, which will in turn reduce the cost of screening, and result in more promising results.

Phylum	% of Total Samples
Porifera	59 %
Cnidaria	14 %
Urochordata (Ascidians)	5%
Echinodermata	5%
Mollusca	2 %
Bryozoa	1%
Crustacea	1%
Miscellaneous	1%
Cyanophyta	1%
Chlorophyta	4%
Phaeophyta	3%
Rhodophyta	5%

Figure 5 Distribution of Organisms in the HBOI Repository by Phylum or Division (Wright 2000)

CHAPTER TWO: MATERIALS AND METHODS

HBOI Peak Fraction Library

We received fractions from HBOI in a 96 well round bottom plate on ice. The plates contained 200 μg of each fraction in the wells. Plates were spun down at 4°C at a speed of 1500 rpm (513 x g) for 5 minutes. After the compounds were spun down, 200 μl of ethanol (200 proof) was added to each well using the epMotion 5070. The plates were then shaken at 4°C for 1 hour to ensure that they were properly mixed. They were then stored at -80°C. Dilutions of the fractions were prepared in ethanol (1:10 and 1:100) and stored at -80°C until they were assayed.

P. falciparum Culture

Two parasite strains were cultured for the duration of this study. The strains used were a chloroquine sensitive strain, 3D7 (Chq^S, Pyr^S, Mef^S, Art^S), and a chloroquine resistant strain, Dd2 (Chq^R, Pyr^R, Mef^R, Art^S). These strains were cultured at 37°C in a humidified environment consisting of 5% CO_2, which has been shown to be an adequate gas supply for *P. falciparum* growth (Radfar, Mendez et al. 2009) . The standard culture media used for maintenance was RP0.5A media (1X RPMI 1640 with L-glutamine supplemented with 0.2% (w/v) dextrose, 15 mg/L hypoxanthine, 25 μg/ml gentamycin, 25 mM HEPES, 0.2% $NaHCO_3$ (w/v), 0.5% (w/v) Albumax.). Whole, A^+ blood was obtained from the Florida Blood Bank. The blood was washed in 1X RPMI-1640 and the buffy coat was removed. The blood was then diluted to 50% RBCs with phenol red-free RP0.5A culture media. Slides were made every day to assess the parasitemia and stage of growth before splitting the culture to a lower parasitemia. Slides were stained using Wright's eosin methylene blue solution and buffer, which gives comparable results to Giemsa stain (Radfar, Mendez et al. 2009).

P. falciparum Synchronization

Parasites were synchronized by two different methods. The first method is a modified version of the protocol described by Lambros and Vanderberg (Lambros and Vanderberg 1979). Cells were synchronized using 5% D-sorbitol which lyses cells in trophozoite and schizont stage. The culture was also synchronized by a gradient method using Percoll solution (65% Percoll in 5X RPMI). Percoll synchronization utilizes a gradient method to distinguish between schizont stage parasites and earlier stage and uninfected RBCs (Saul, Myler et al. 1982; Dluzewski, Ling et al. 1984). Infected RBCs are evaluated to ensure that a majority of the parasite culture is in the schizont stage. Culture is then prepared in a 20% cell suspension in sterile 1X RPMI and layered onto the Percoll solution. Cells are then spun down at 2000 rpm (911.5 x g) for five minutes at room temperature and the schizont layer is removed and placed in a new culture dish with fresh media and blood.

Hypoxanthine Incorporation Assay

Asynchronous culture was used for the [^{3}H]-hypoxanthine incorporation and SYBR Green-I based assays. During the [^{3}H]-Hypoxanthine incorporation assay, culture was added to a 96 well clear plate at a 1% parasitemia and 2% hematocrit. The media used during this assay was RP0.5A with low hypoxanthine, 2.5 mg/L hypoxanthine. The culture was then supplemented with [^{3}H]-Hypoxanthine at a concentration of 5 μCi/mL, and then 100 μl of the radioactive culture was added to each well. The culture was then incubated with the HBOI peak fractions at the appropriate concentrations for 72 hours, enough time for the parasites to complete 1.5 life cycles. The cells were then harvested using the TOMTEC cell harvester onto a glass fiber mat. Infected RBC was lysed by freezing before the labeled nucleic acid was deposited on the glass fiber mat. Each of the squares, corresponding to individual culture wells, on the mat were then

cut and placed into individual scintillation vials and suspended in ScintiVerse BD cocktail from Fischer Scientific. The vials were then read on the scintillation counter to obtain the amount of radioactivity present within each well.

SYBR Green-I Fluorescence Assay

SYBR Green-I based fluorescence assay were performed in a 96 well plate format. HBOI fractions, at their desired concentrations, were incubated with 100 μl of culture at a 1% parasitemia and a 2% hematocrit. Culture was added to each well using the epMotion 5070 liquid handler. The plates were then incubated for 72 hrs at the standard gas and temperature conditions. Once the 72 hour incubation was completed, the parasite culture was frozen at $-80^{\circ}C$, thawed, and lysed with a 1X SYBR Green-I dye containing lysis buffer (20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% Saponin (wt/vol), 0.08% TritonX-100 (vol/vol)). The plates were next incubated at 37°C for 30 minutes, and then read on Wallac plate reader Victor[2]- 1420 multilabel counter. Results were analyzed using Microsoft Excel and GraphPad Prism software. The controls used in the [3H]-hypoxanthine incorporation assay as well as the SYBR Green-I fluorescence assay were 1 μM Chloroquine and 1 μM Artemisinin for 3D7 and Dd2 lines, respectively.

Assay Accuracy and Sensitivity

Originally developed to validate hits from the high throughput screening of chemical libraries, the Z-factor is defined as the screening window coefficient. The Z-factor is calculated by $Z' = 1 - 3((\sigma_{c+} - \sigma_{c-})/ |\mu_{c+} - \mu_{c-}|)$. The value represents the degree of sensitivity and accuracy of the assay being used. When the Z-factor is equal to 1, the assay is said to be ideal. A value below 1, but greater than 0.5 represents an excellent assay. Whereas values below 0.5 indicates that the assay is not accurate, and another method should be identified for further screening (Zhang,

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Chung et al. 1999). The mean values and standard deviations were calculated using Microsoft Excel software.

Mammalian Cell Toxicity Assay

The NIH 3T3 human fibroblast cell line was used in the presence of HBOI peak fractions to determine the selectivity index. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM sodium bicarbonate, 1X Pen-strep, and 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified, 5% CO_2, environment. In order to maintain the cell lines, cells were seeded into a new dish once they reached 90% confluency. During this step, the cells were washed with 1X DPBS, followed by treatment with 1X Trypsin-EDTA for thirty seconds and a two minute incubation at 37°C after the liquid was removed to release the cells from the surface of the dish. The dish was then placed at 37°C and incubated for two minutes before they were resuspended in DMEM and added to a fresh culture flask.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Viability Assay, was performed following an adapted protocol outlined by Mosmann (Mosmann 1983). To begin, viable cells were counted using the trypan blue (Sigma-Aldrich) exclusion method. Cells were then counted on a hemacytometer, and diluted to 15,000 viable cells per 50 μl of phenol red-free culture media. Cells were then pipetted into a 96-well clear plate and incubated for 10-12 hours to allow the cells to reattach to the surface of the plate. While the cells were incubating, the selected peak fractions were pipetted out into microcentrifuge tubes at the appropriate concentrations. The ethanol was allowed to evaporate out of each tube. Once the ethanol had evaporated out of each tube, the fraction was resuspended in phenol red-free DMEM. Before the fractions were added, their UV absorbance was measured on the Nanodrop 1000. After the tube

was empty and the contents were added to each well, the same volume of ethanol (200 proof) was added to the microcentrifuge tube. The UV absorbance was then read again to ensure that the all of the compound was removed from the tube. The plates were then incubated with the fractions at 37°C for 15 hours. After 13 hours of incubation, 60 ng/ μl digitonin was added to the control wells, and the plate was placed back in the 37°C incubator. Once the cells completed their 15 hour incubation, 20 μl of MTT reagent (5 mg/ml) was gently added to each well. The plate was incubated for an additional 4 hours at 37°C. Without disturbing the bottom of the well, the MTT reagent and media were removed from the wells, and 150 μl of MTT Solvent (Isopropanol, 4 mM HCL, 0.1% Nonidet P-40) was added to each well. The plate was then covered with aluminum foil and shaken on the Eppendorf MixMate for 15 minutes. The absorbance was then read on the Tecan Infinite M200 Plate reader at 590 nm, with a reference reading at 700 nm. Results were analyzed using Microsoft excel and Graph Pad Prism Software.

CHAPTER THREE: SCREENING OF HBOI PEAK FRACTION LIBRARY

To identify new leads for malaria therapy we have screened HBOI's peak fraction library collection. A rapid turnaround time is needed to efficiently screen these libraries and is essential to the success of this project. The current standard for screening new potential antimalarials in *Plasmodium falciparum* species is through the use of a [^{3}H]-hypoxanthine incorporation assay. This method has proven to be a reliable screening method, but needs radioactive material for the assay. This results in a lower assay throughput, and a higher cost of around $33.00 per assay (Abiodun, Gbotosho et al. 2010). An alternative method utilizes the DNA intercalating fluorescent dye SYBR Green-I to stain parasite DNA within mature RBCs. SYBR Green-I dye has an affinity for staining double stranded DNA as opposed to ssDNA and RNA(Johnson, Dennull et al. 2007) . This allows it to be a useful tool in the screening process because the only DNA that will be monitored is that of the parasite present within the infected RBC (iRBC). The usefulness of the SYBR Green-I assay is that it can be developed into a 96-, 384-, or 1536- well format. For example, recently, libraries of 1.7 million compounds have been screened in a 1536 well format and yielded around17,000 potent inhibitors of *Plasmodium* (Plouffe, Brinker et al. 2008). The use of SYBR Green-I as a primary screening tool has been validated by many laboratories, and has become a reliable, low cost, around $1.75 per assay (Abiodun, Gbotosho et al. 2010), method in high throughput *P. falciparum* screening (Bennett, Paguio et al. 2004; Johnson, Dennull et al. 2007; Rason, Randriantsoa et al. 2008; Co, Dennull et al. 2009). We tested both the [^{3}H]-hypoxanthine and SYBR Green-I assay methods before engaging into a large scale screening of the HBOI peak fraction library in a 96-well plate format to identify novel antimalarials.

Validation of SYBR Green-I Fluorescence based assay

[3H]-Hypoxanthine incorporation assays have become an extremely reliable method for *in vitro* growth assessment in *P. falciparum* culture. This is due to the fact that *Plasmodium spp.* of parasites are unable to synthesize purine rings, relying on a salvage pathway instead for de novo synthesis (Downie, Kirk et al. 2008).In order to compare the [3H]-Hypoxanthine incorporation assay with the SYBR Green-I fluorescence based assay, we analyzed the Z-factors and quantity of hits identified. Our results show that the Z-factors for both assays were above the required 0.5 (Zhang, Chung et al. 1999), with the [3H]-Hypoxanthine incorporation assay having a Z-factor of 0.69, and the SYBR Green-I Fluorescence based assay having a Z-factor of 0.52. Although the [3H]-Hypoxanthine incorporation assay had a higher Z-factor, both assays identified the same initial hits from the peak fraction library having similar percent inhibition levels. Based on this result, we decided to continue screening using the SYBR Green-I (SG) fluorescence based assays for our screening because of the ease of using a non-radioactive assay.

Primary Screening of Marine Natural Products

Once the reliability of the SG assay was established, the remaining HBOI Peak library plates were screened against the chloroquine sensitive strain of *P. falciparum* 3D7 strain. Each of the thirty-three HBOI peak fraction library plates were plated at a concentration of 10 μg/mL, and incubated with 3D7 *P. falciparum* culture for 72 hours. The duration of incubation is enough to allow the parasites to complete one and half life cycles. This will also ensure that the fractions will have an opportunity to inhibit the growth during every stage of development, as well during the invasion of the parasite into the RBC at the beginning of the next developmental cycle.

During the primary screening we identified a total of 253 fractions out of 2,830 that inhibited parasite growth at greater than or equal to 70% of the controls. This represented an

initial hit rate of 8.94%, with an average Z-factor of 0.77 (Table 1). From the positive fractions, we selected the two best inhibiting fractions from each species of marine organisms for further analysis. Due to the rate at which new plates were received, primary focus was placed on the MNP plates Mal004 through Mal011. Mal004 through Mal011 were screened for their percent inhibition at 5 µg/mL (Table 2), and those that were found to inhibit growth at greater than or equal to 70% of the control were selected as preliminary hits. Hits are generally defined as those products whose IC_{50} values are less than 0.2 µg/mL of pure compound in *P. falciparum*. The selection criteria for our screening were adjusted because fractions, although enriched, are likely to contain multiple chemical compounds. Therefore, fractions were selected based on the criteria stated above with the initial screening criteria being left up to the discretion of the primary investigators (Fidock, Rosenthal et al. 2004; Nwaka and Hudson 2006).

The IC_{50} value for each of the selected fractions was determined using the SG assay to measure parasite growth. The average Z-factor calculated for these assays were 0.76, indicating that the assays were statistically reliable (Table 3). Table 3 represents the fractions whose IC_{50} values were below 5 µg/mL, with fifteen of the thirty-three fraction's IC_{50} values being less than 1 µg/ml. Dose response graphs representing the potency of a few selected peak fractions are shown in figure 6. The four fractions represented are from four different marine organisms whose IC_{50} values were below 1.5 µg/mL. The fractions that had an IC_{50} value less than 5 µg/mL were selected to further investigate their potency against the chloroquine resistant strain of *P. falciparum*.

Plate ID	SYBR Green at 10 μg/ml	Plate ID	SYBR Green at 10 μg/ml
Mal 004	6 fractions	Mal 005	3 fractions
Mal 006	3 fractions	Mal 007	5 fractions
Mal 008	5 fractions	Mal 009	9 fractions
Mal010	18 fractions	Mal011	29 fractions
Mal012	9 fractions	Mal013	11 fractions
Mal014	5 fractions	Mal015	21 fractions
Mal016	9 fractions	Mal017	12 fractions
Mal018	17 fractions	Mal019	4 fractions
Mal020	13 fractions	Mal021	9 fractions
Mal022	6 fractions	Mal023	4 fractions
Mal024	7 fractions	Mal025	1 fraction
Mal026	4 fractions	Mal027	7 fractions
Mal028	5 fractions	Mal029	6 fractions
Mal030	7 fractions	Mal031	6 fractions
Mal032	6 fractions	Mal033	6 fractions

Table 1 The number of peak fractions inhibiting chloroquine sensitive 3D7 growth. Each fraction that was identified as a preliminary hit based on the percent inhibition compared to 1 μM chloroquine.

Unique I.D	% Inhibition at 5μg/ml	Unique I.D	% Inhibition at 5μg/ml	Unique I.D	% Inhibition at 5μg/ml
4.A06	83.0	7.G05	111.0	9.G07	96.0
4.A10	80.0	7.G11	108.0	9.H09	107.0
4.B03	86.0	7.H01	104.0	10.A02	104.0
4.B07	84.0	8. E04	80.0	10.C10	98.0
4.H01	79.0	8. E06	90.0	10.C11	96.0
5.B05	88.0	8. E08	106.0	10.D08	95.0
5.G06	103.0	8.F01	88.0	10.D09	83.0
5.G07	109.0	8.F07	83.0	11.A08	87.0
6.D04	78.0	9.C06	95.0	11.B03	92.0
6.H04	84.0	9.C07	90.0	11.H01	96.0
7.C08	96.0	9.G04	95.0	11.H02	98.0

Table 2 Inhibiting peak fractions from plates Mal004 through Mal011. Inhibition was measured in comparison with the inhibition of parasite growth by 1 μM chloroquine in chloroquine sensitive parasites.

Unique I.D	IC$_{50}$ μg/ml	Unique I.D	IC$_{50}$ μg/ml	Unique I.D	IC$_{50}$ μg/ml
4.A06	1.5	7.G05	0.4	9.G07	0.2
4.A10	2.2	7.G11	< 0.25	9.H09	0.5
4.B03	2.4	7.H01	1.7	10.A02	2.0
4.B07	0.9	8. E04	1.3	10.C10	2.2
4.H01	0.9	8. E06	1.5	10.C11	0.2
5.B05	1.2	8. E08	1.8	10.D08	< 0.2
5.G06	1.3	8.F01	0.6	10.D09	1.1
5.G07	4.8	8.F07	<0.25	11.A08	1.1
6.D04	0.8	9.C06	<0.25	11.B03	0.7
6.H04	1.4	9.C07	<0.25	11.H01	1.3
7.C08	4.6	9.G04	2.5	11.H02	0.8

Table 3 IC$_{50}$ values of inhibiting peak fractions in 3D7. The IC$_{50}$ values for the preliminary hits were determined using the SG assay with 1 μM chloroquine as a control for parasite growth inhibition.
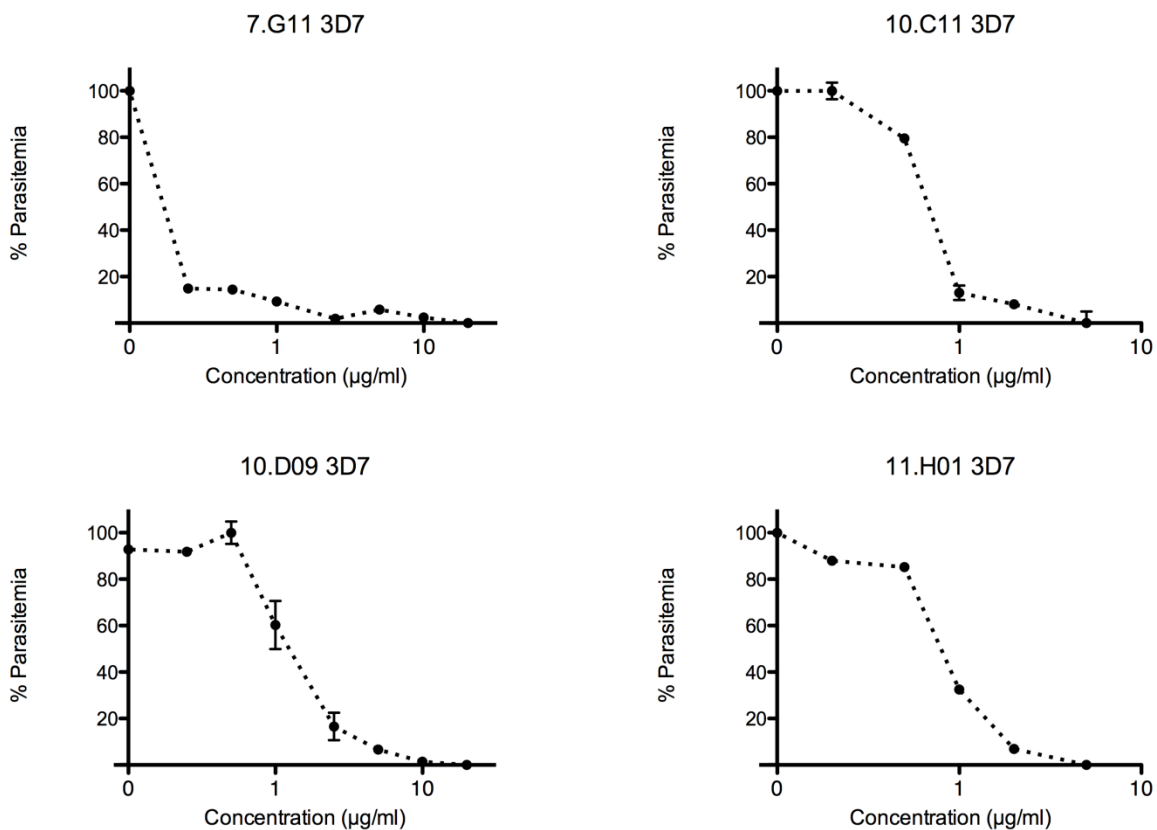
Figure 6 Dose-response curves were used to evaluate the IC_{50} value of preliminary hits. Percent parasitemia was determined by the comparison of parasite growth in treated parasites versus those treated with 1 μM chloroquine, and those that remained untreated.

<u>Screening of Peak Fractions against Drug Resistant *P. falciparum*</u>

Any effort to find a novel cure for malaria must demonstrate that the active compound is also equally effective against chloroquine resistant strains. To address this issue, peak fractions were also screened against Dd2, the laboratory chloroquine resistant strain (Chq^R, Pyr^R, Mef^R, Art^S). This allows us to predict which of the fractions will be more useful against drug resistant malaria.

We determined the percent inhibition at 5 μg/mL (Table 4) and IC_{50} values (Table 5) against the chloroquine resistant strain. Our initial screening against Dd2 at 5 μg/mL, showed that 82% of the peak fractions that were active against the sensitive 3D7 strain inhibited Dd2 growth at greater than or equal to 70% of the control, 1 μM artemisinin. The average Z-factor from these screening efforts was 0.75, making these results statistically reliable. The IC_{50} values of the inhibitory peak fractions were determined using the Dd2 strain. Among the thirty-three inhibitory fractions, twenty five fractions exhibited IC_{50} values less than 5 μg/mL against chloroquine sensitive and resistant strains, 3D7 and Dd2 respectively. Of the twenty-five that were found to be inhibitory, the fractions derived from four species, *Axinellida*, *Spirastrella sp.*, *Gastropoda Aplysia sp.*, and *Peysonellia*, were found to be the most potent. The peak fractions from these species had IC_{50} values less than, or equal to, 1.1μg/mL (Figure 7). These fractions were selected for further investigation of their potential cytotoxic properties in human fibroblast cells.

Unique I.D	%Inhibition at 5 µg/ml	Unique I.D	%Inhibition at 5 µg/ml	Unique I.D	%Inhibition at 5 µg/ml
4.A06	103.9	7.G05	103.3	9.G07	99.0
4.A10	74.9	7.G11	105.0	9.H09	94.7
4.B03	77.5	7.H01*	65.4	10.A02	99.9
4.B07	76.6	8. E04*	43.8	10.C10	105.9
4.H01	101.9	8. E06	95.3	10.C11	101.0
5.B05	69.7*	8. E08	89.7	10.D08	99.2
5.G06	106.0	8.F01	70.2	10.D09	82.7
5.G07	102.3	8.F07	72.1	11.A08	91.7
6.D04	79.7	9.C06*	49.0	11.B03	95.5
6.H04	101.5	9.C07*	55.9	11.H01	104.0
7.C08	10.4*	9.G04	99.4	11.H02	107.8

Table 4 Inhibition of chloroquine resistant parasites. A Dd2 parasite cell line was used to screen the preliminary hits for their ability to inhibit compared to 1 µM artemisinin. Inhibition of Dd2 percent inhibition was then compared to 3D7 percent inhibition to predict which fractions had the potential to work in resistant strains of the parasite.

Unique I.D	IC$_{50}$ µg/ml	Unique I.D	IC$_{50}$ µg/ml	Unique I.D	IC$_{50}$ µg/ml
4.A06	1.3	**7.G11**	0.5	**10.A02**	1.1
4.A10	2.3	**8. E06**	4.3	**10.C10**	0.7
4.B07	1.0	**8. E08**	2.4	**10.C11**	0.2
4.H01	4.8	**8.F01**	4.9	**10.D08**	0.1
5.G06	4.2	**8.F07**	4.9	**10.D09**	<0.2
5.G07	3.1	**9.G04**	2.0	**11.A08**	2.5
6.H04	2.1	**9.G07**	0.8	**11.B03**	2.6
7.G05	1.5	**9.H09**	4.6	**11.H01**	1.1
				11.H02	0.8

Table 5 IC$_{50}$ values of inhibiting peak fractions in Dd2. The IC$_{50}$ values were determined using the SG assay with 1 µM artemisinin as a control for inhibition of chloroquine resistant parasite growth.
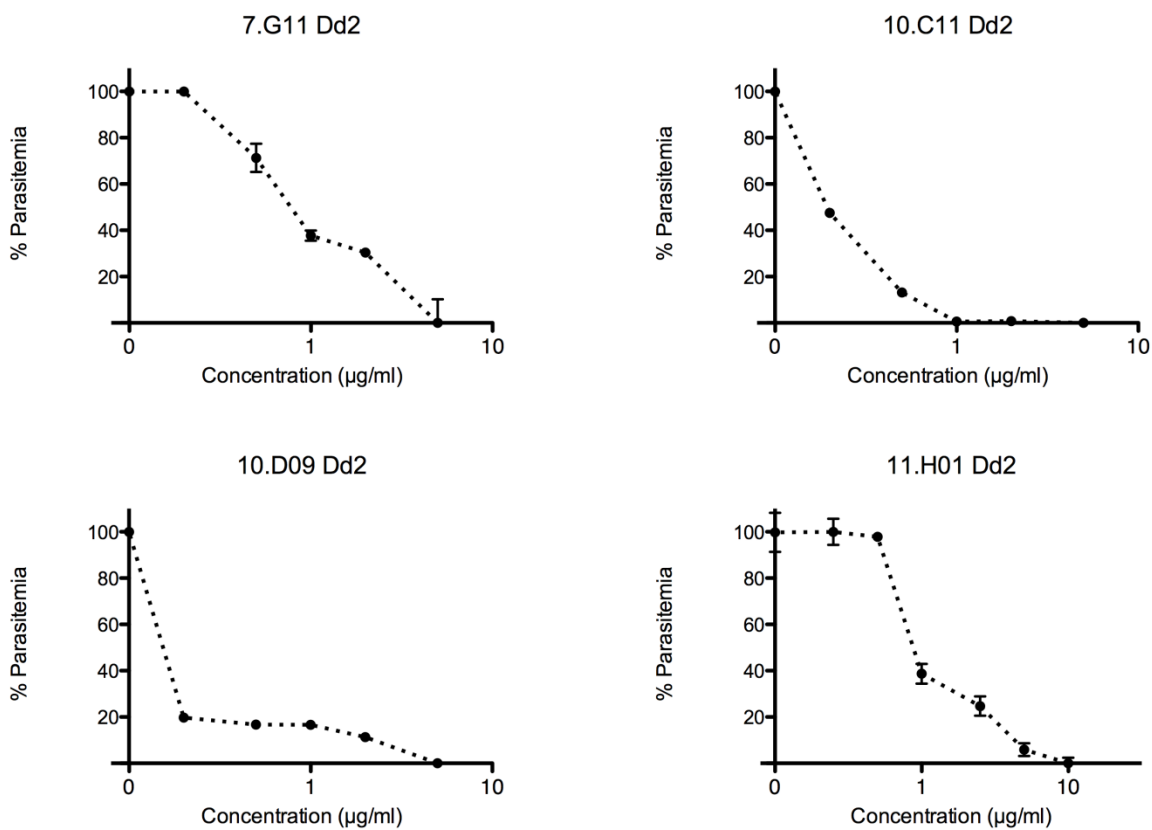
Figure 7 Dose-response curves were used to evaluate the potency of preliminary hits against chloroquine resistant parasites. Percent parasitemia was determined by the comparison of parasite growth in treated parasites versus those treated with 1 μM artemisinin, and those that remained untreated.

CHAPTER FOUR: MTT CELL VIABILITY ASSAY

One of the most significant issues in drug discovery research is the rate of failure. For every ten compounds that make it to phase I clinical trials there is only one that can be marketed as a drug. Reasons for failure are in many circumstances related to the safety of a drug in patients (Bass, Cartwright et al. 2009). Therefore, an important criterion for selecting a bioactive fraction for lead discovery is to determine its selectivity index. As a potential antimalarial, the positive fraction we have identified must show that it inhibits the growth of *P. falciparum* without causing harm to the human host. In order to eliminate potential toxicity issues, researchers have begun to use a variety of screening techniques to predict the *in vivo* selectivity profile of lead compounds (Bass, Cartwright et al. 2009). Using these results, the most selective and bioactive compounds are used for further studies. Two commonly used cell viability assays were used to screen against active peak fractions to eliminate those that could potentially be cytotoxic. One of the first attempts to study cell viability was performed using the MultiTox-Glo Cytotoxicity assay commercially available from Promega. Protease markers are identified in viable cells and dead cells using two different protease substrates which become fluorescent or luminescent when interacting with its protease marker (Niles, Moravec et al. 2007). Viable cells are measured based on the amount of substrate that interacted with the Cathepsin-C, a viable cell protease, while dead cells are measured by the substrate which interacted with Tripeptidyl peptidase II, an active protease during apoptosis (Niles, Moravec et al. 2007). Ideally, this method would have given results that inversely corresponded to one another. We performed the assay according to the protocol outlined by Promega with NIH 3T3 fibroblast cells and found that the results were not reproducible; therefore we began to look for alternative methods for evaluating cell viability. Rather than looking at specific markers for protease activity, we chose to use the MTT assay

which measures cellular metabolism. Tetrazolium dyes are reduced by mitochondrial dehydrogenases present in living cells to a blue formazan crystal. Using this method, crystals are dissolved in an isopropanol based solvent, and the absorbance corresponds to the amount of living cells present within each well (Mosmann 1983). This assay is very reliable, and when compared to the MultiTox-Glo Cytotoxicity assay (around $180.00 per 96-well plate assay), is less expensive. For those reasons, we chose to use the MTT assay with a human fibroblast cell line, NIH 3T3 to study the selectivity index of potent MNPs. The NIH 3T3 cell line has been successful in previous cell viability assays (Theiszova, Jantova et al. 2005).

<p style="text-align:center"><u>HBOI Peak Fraction Library Selected Fractions</u></p>

The peak fractions from the top four inhibiting species identified during the initial screening of the HBOI peak fraction library plates Mal004 through Mal011 were screened against NIH 3T3 fibroblast cells to assess their cytotoxic properties and determine their selectivity index, a comparison of peak fraction IC_{50} values between *Plasmodium* and fibroblast cultures. Cells were incubated with each peak fraction for 15 hours at 3 different concentrations, 1 μg/mL, 10 μg/mL, and 100 μg/mL (Figure 8). These graphs showed that five out of the eight fractions exhibited selectivity indices ten, or greater. Three of the fractions, 10.D08, 11.H01, and 11.H02, were found to exhibit cytotoxic properties against the fibroblast cells, with selectivity indices below ten. Knowing their potential cytotoxic risk, these fractions will be eliminated in future studies of these MNPs.
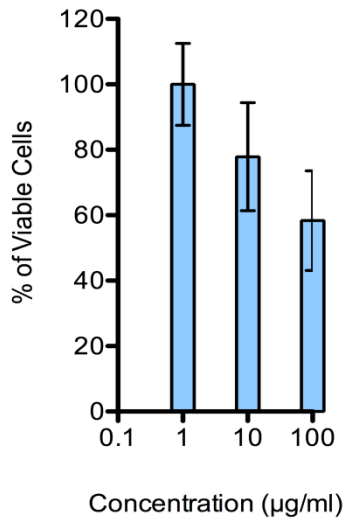
7.G11
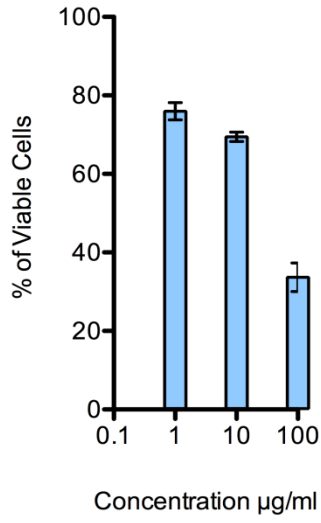
9.G07 (37.A09)
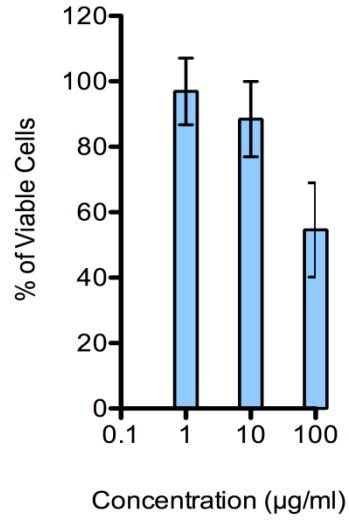
10.C10 (37.A11)

10.C11
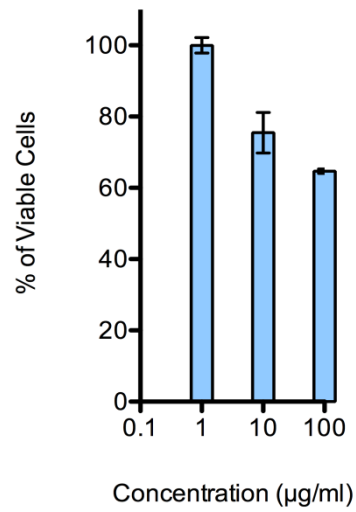
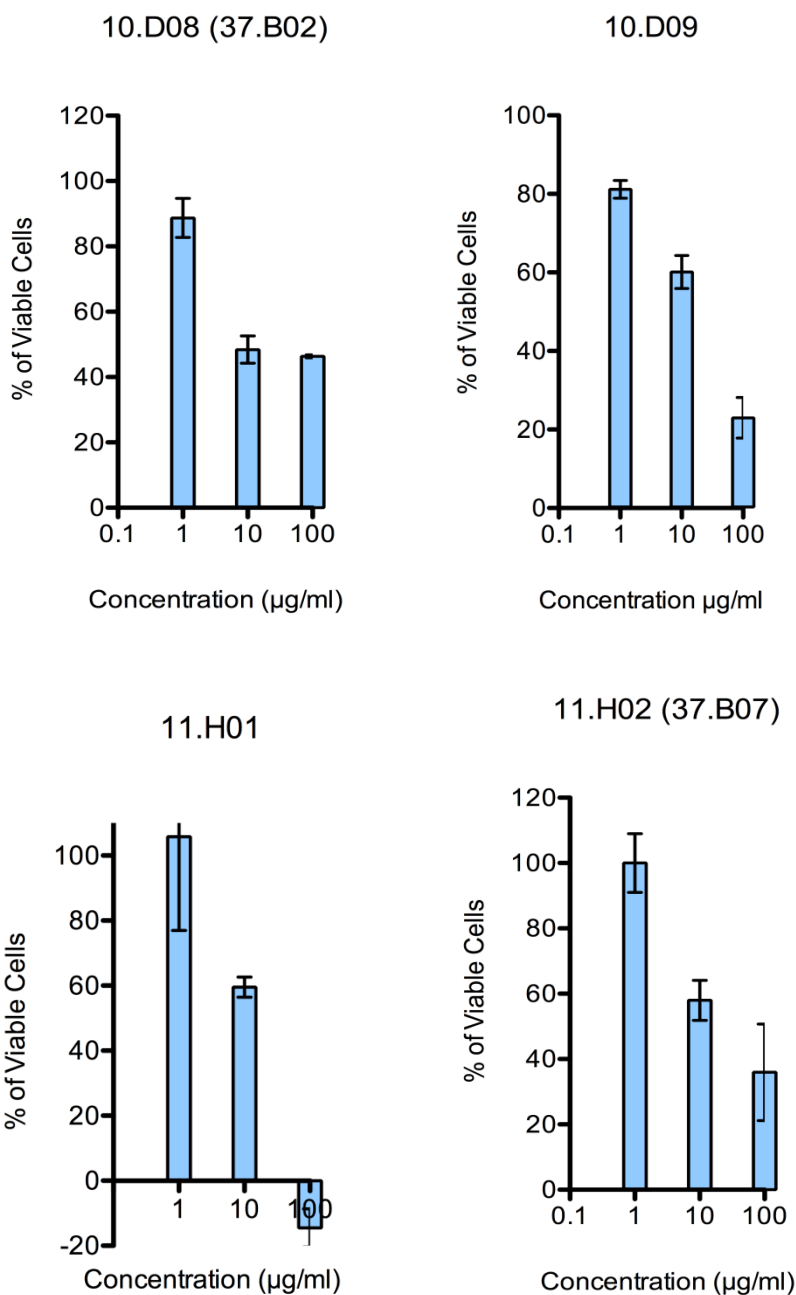Figure 8 Toxicity profiles of select MNPs exhibit promising selectivity indices. Prediction of *in vivo* selectivity index was performed by comparison of inhibition in the presence of selected peak fractions compared to cells that were untreated and those that were treated with digitonin. Peak fractions that had a selectivity index greater than 10 were determined to be promising for future studies.

CHAPTER FIVE: NORTOPSENTIN A AS A NOVEL ANTIMALARIAL

Nortopsentin A inhibition of *Plasmodium falciparum* growth

HBOI's peak fraction library consists of enriched fractions that contain more than one compound. The separation of single compounds requires further purification steps which are performed at HBOI. Once the fraction has been purified and the chemical structure has been identified, the information is sent to our lab where we screen the fraction again for its molar IC_{50} value in chloroquine sensitive and chloroquine resistant strains. Previous screening of the MNP peak fraction library revealed fractions from the *Spongosorite* species of sponges to be potent inhibitors of chloroquine sensitive parasite growth. Upon purification of extract from this species of sponge, Nortopsentin A was one of the chemical scaffolds identified (Figure 9). Nortopsentin A has been identified and screened against a variety of tumor cell line, as well as screened for its anti-inflammatory properties (Wright 2000; Diana, Carbone et al. 2007). When searching through the literature, it was found that Nortopsentin A had not yet been identified as an antimalarial, making it an attractive compound to continue for further studies.

Using the SG assay, we determined the IC_{50} value for Nortopsentin A in both 3D7 and Dd2. These values were identical in both strains of *P. falciparum* screened (Figure 10). Both values were found to be 1.6 μM, which is within the accepted antimalarial standard IC_{50} value range of 1 to 5 μM (Fidock, Rosenthal et al. 2004). The Z-factors for both assays were 0.95, making these results statistically reliable.

Nortopsentin A Cell Viability Assay

As with the aforementioned cell viability assays, an important step in the process of discovering a novel antimalarial, is the determination of the selectivity index to eliminate possible failures during *in vivo* studies. In order to ascertain this data, the compound was tested

at 0.5X, 1X, 10X, 100X, and 200X the IC$_{50}$ value calculated for its activity against *P. falciparum*

(Figure 11). Results from this assay indicated that Nortopsentin A exhibited cytotoxicity at high

concentrations in the fibroblast cells, with its IC$_{50}$ value in fibroblast cells being 15 μM, a
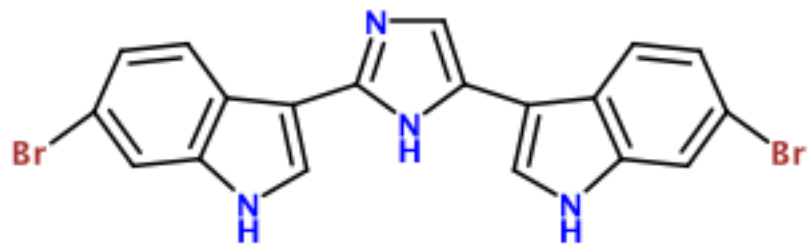
selectivity index of 9.4.

Figure 9 Chemical structure for Nortopsentin A.
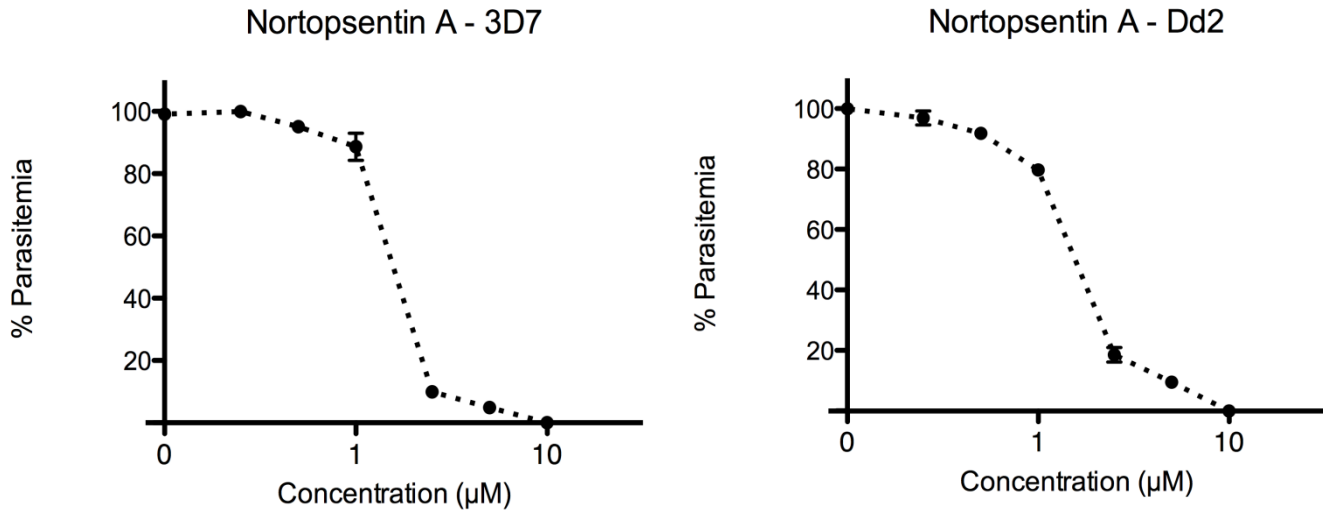
Figure 10 Inhibition of chloroquine sensitive and chloroquine resistant parasite growth by Nortopsentin A. Dose-dependent inhibition was performed beginning at 10 µM concentration and decreasing to 0.2 µM. The assay was performed using the 1 µM concentrations of chloroquine and artemisinin as positive controls.

Figure 11 Toxicity in the presence of Nortopsentin A. Toxicity was evaluated at concentrations of 0.5X, 1X, 10X, 100X, and 200X of the parasite IC_{50} value, using the absence of drug treatment and digitonin treatment as positive and negative controls.

Effect of Nortopsentin A on Parasite Development

In order to better understand the mechanism of action on *Plasmodium*, 3D7 culture was incubated with 3 μM Nortopsentin A and time points were collected. Giems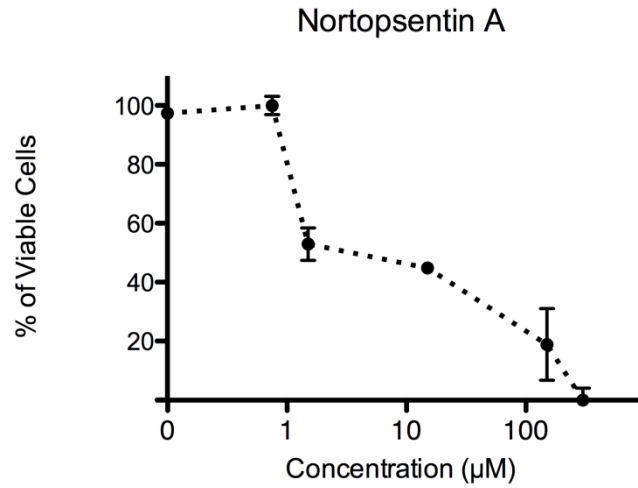a stained slides were made at 16 hours, 32 hours, and 48 hours. The parasite stages in the presence of Nortopsentin A and those without any inhibitor were then compared using a light microscope (Figure 12). Our results indicated that Nortopsentin A inhibited parasite growth at this concentration and acted on the early stage of parasite development. The parasites in the absence of inhibitor progressed through each stage of development from ring to schizont to re-infection. In contrast, the development of the parasite in the presence of Nortopsentin A at 3μM was blocked at the late ring/early trophozoite stage.
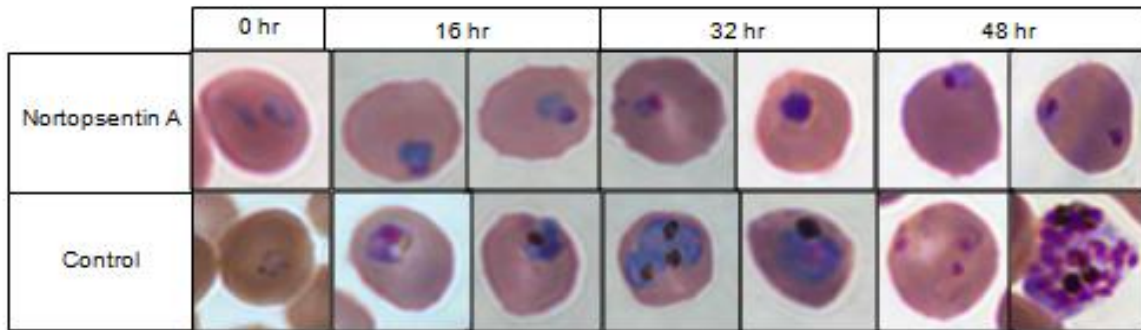
Figure 12 Development of chloroquine sensitive parasites in the presence of 3 μM Nortopsentin A. Images of the parasite culture were taken at 0 hours, 16 hours, 32 hours, and 48 hours. The stage of growth was observed in both treated and untreated cells to determine the effect of Nortopsentin A on parasite development.

CHAPTER SIX: DISCUSSION

Plasmodium falciparum is the deadliest form of parasite that causes malaria infection. The emergence of drug resistant strains has led to a resurgence of drug discovery research in an effort to find novel, safe, effective, and inexpensive therapies and prophylactics. Using the peak fraction library supplied by HBOI, we have successfully screened over 2,000 marine natural products for their antimalarial activity. With the use of the SYBR Green-I fluorescence based assay, we have screened these fractions quickly and accurately, without the inconvenience of using radioactive material. Using this assay we found there to be a nine percent hit rate, which is higher than the hit rate of 0.3% and 0.001% for the average natural product and synthetic library (Li and Vederas 2009). Screening of HBOI's peak fraction library has led to many options for future studies. Identifying a larger percentage of biologically diverse preliminary hits will hopefully lead to a higher rate of success later in the drug discovery pipeline.

Peak fractions that we screened represented a diverse number of marine organisms, around 122 different species. The organisms that are present within the library are unrelated to many terrestrial plants, and therefore hold the potential for containing novel chemical scaffolding for drug discovery (Gul and Hamann 2005). Some of the more promising species from which fractions originated included sponges, *Axinellida* and *Spirastrellla*, algae, *Peysonellia*, and a species of mollusks, *Gastropoda Aplysia*. Two of the more potent and least cytotoxic fractions originated from *Spirastrella*. It has been previously reported that this sponge is capable of producing secondary metabolites that act as antimitotic and antiviral drugs (Garg and Agrawal 1995; Williams, Roberge et al. 2003). One of the most potent fractions screened was isolated from the species *Peysonellia*, an alga species, and was previously reported to act as an anticancer drug (Crews, Gerwick et al. 2003). As described, these are only two of the 122 species that we

identified as preliminary hits. Having potent antimalarial activity and promising cytotoxic profile, purification followed by structure identification of these fractions will allow us to identify novel antimalarials.

Only one pure compound, Nortopsentin A, was analyzed in this study. This compound was isolated from a species of sponges, *Spongosorites*, and is a potent inhibitor of drug susceptible and resistant *P. falciparum* growth, with around a tenfold difference between the IC_{50} values in NIH 3T3 fibroblast cells and *P. falciparum* culture. It is a bis(indolyl) alkaloid which has been found to be within a group of sponge metabolites that are recognized for their anti-inflammatory, antimicrobial, antiviral, and antitumor activities at low IC_{50} values (Diana, Carbone et al. 2007). With the discovery of its antimalarial activity, we wanted to determine the potential mechanism of action for this compound and were able to clearly see that not only is the parasite growth inhibited, but the development of the parasite into more mature stages is halted between late ring and early trophozoite stage (Figure 10). Previous studies of alkaloids have shown that they can act as DNA intercalating agents and halt DNA synthesis (Martinez and Chacon-Garcia 2005). Our results correlate well with the known mechanism of Nortopsentin A as DNA synthesis occurs during the trophozoite stage of the asexual, erythrocytic growth. If Nortopsentin A binds to DNA during ring stage of development, DNA synthesis in trophozoite stage will likely be inhibited, and progression of the cell cycle would cease. Further studies are needed to fully understand the mechanism by which Nortopsentin A inhibits the growth of *P. falciparum*. Although Nortopsentin A is the most promising hit we have identified, its current profile, shows that it is a potent inhibitor of *P. falciparum*, but the projected selectivity index needs to be increased to greater than 100 by structural modifications in order to be considered as a candidate for lead optimization (Fidock, Rosenthal et al. 2004).

Marketing a new antimalarial requires that a compound be potent against the parasite, along with having favorable selectivity and pharmacological properties. Structural modifications are necessary to obtain a marketable product, and require the assistance of medicinal chemist to view the structural properties and create a series of modified structures. For Nortopsentin A, modified structures have already been synthesized for other therapeutic purposes. Some of these modifications include the bromination of the R group in Nortopsentin's structure. The addition of this group has been shown to decrease activity in a leukemia cell line, P-388 (Gul and Hamann 2005). When analyzing the potency of these modified structures, the pharmacological properties of the compound must also be considered. Structural modifications need to be made to ensure that Nortopsentin A is soluble, that it is not metabolized quickly, and that it does not bind to plasma. In order ascertain this information, an ADME (absorption, distribution, metabolism, and excretion) profile of the compound can be developed through a series of laboratory experiments. With these modified structures already synthesized, we can compare the activities, toxicity, and pharmacological properties, and select the most attractive structure for lead discovery.

Previous studies on the use of MNPs from HBOI were focused on other therapeutic areas of research such as antitumor, antiviral, and antimicrobial agents; however, their ability to collect fractions from novel sources and perform structural identification has allowed us to create a library of potential, novel antimalarial leads. Here, we have shown that through the screening of a novel peak fraction library, we can identify potential lead compounds..

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