Identification Of Novel Antimalarial Scaffolds From Marine Natural Products

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IDENTIFICATION OF NOVEL ANTIMALARIAL SCAFFOLDS FROM MARINE NATURAL PRODUCTS

by

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ABSTRACT

Malaria, the disease caused by *Plasmodium sp.*, claims the lives of over 1 million people every year, with *Plasmodium falciparum* causing the highest morbidity. Rapidly acquiring drug resistance is threatening to exhaust our antimalarial drug arsenal and already requires the utilization of combination drug therapy in most cases. The global need for novel antimalarial chemical scaffolds has never been greater.

Screening of natural product libraries is known to have higher hit rates than synthetic chemical libraries. This elevated hit rate is somewhat attributed to the greater biodiversity available in natural products. Marine life is the most biodiverse system on the planet, containing 34 of the 36 known phyla of life, and is expected to be a rich source of novel chemotypes. In collaboration with the Harbor Branch Oceanographic Institute in Ft. Pierce we have screened a library of over 2,800 marine macroorganism peak fractions against *Plasmodium falciparum* using the SYBR green I fluorescence-based assay. In this screening process we have identified six compounds from five novel chemical scaffolds all of which have low micromolar to submicromolar IC_{50} values and excellent selectivity indices. Additionally, one of these chemical scaffolds, the bis(indolyl)imidazole, was selected for further in vitro pharmacological and structure-activity relationship (SAR) profiling, key steps in the challenging process of identifying a new antimalarial drug lead compound.

I dedicate this work to my friends, the drug discovery lab team, and my wonderful family. Without their support, love, and advice these findings would yet be unknown. Thank you.

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First and foremost I thank my mentor Dr. Debopam Chakrabarti. His demand for excellence has forever changed my research endeavors. I also thank Dr. Roseann White and Mr. Greg Weigel whose kindness and friendship have supported me in my pursuit of this degree.

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CHAPTER ONE: INTRODUCTION

Malaria Background

Malaria, a term from the Latin words 'mal' 'aria' meaning 'bad air', is caused by the parasite genus *Plasmodium* of the apicomplexan family. Once infected the most common symptoms include fever, headache, malaise, myalgias, arthralgia, low back pain, nausea, vomiting, diarrhea and cough [1]. The most prevalent human parasites are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malaria,* and *Plasmodium knowlesi,* with *Plasmodium falciparum* causing the highest global mortality [2]. Despite the fact that Malaria has been a major cause of mortality and morbidity for thousands of years [3], recent estimates conclude that around 3.3 billion people are still at risk of contracting malaria, and over 1,000,000 people die annually from the disease [2]. Over 85% of the mortality occurs in children under 5 years old.

Plasmodium falciparum Life Cycle

The *Plasmodium falciparum* life cycle involves two separate hosts, the female *Anopheles* mosquito and the human. The parasite enters the mosquito during a blood meal as exflagellated motile microgametes and macrogametes [4]. These quickly form male and female gametocytes which undergo gametogenesis to form ookinetes. The ookinetes then migrate to the mid-gut epithelium basal lamina [5], where the ookinetes develop into oocytes and mature into sporozoites that rupture through the basal lamina and migrate to the mosquito salivary gland [6]. Upon the mosquito's next blood meal, sporozoites travel from the mosquito salivary gland and into the human blood stream. Within 30 seconds to two minutes the sporozoites must travel along the human blood stream and terminally lodge within hepatocyte cells where they will

replicate to over 10,000 merozoites in a process called schizogony [7, 8]. Eventually, a merosome, full of hundreds of merozoites, will bud and release the merozoites inside the blood stream, each of which will infect a red blood cell [9]. Over the next 48 hours the intra-erythrocytic merozoite will develop into a ring, then a trophozoite, followed by schizont. Within the schizont stage the parasite undergoes many consecutive cycles of replication forming a 10-20 nucleated merozoites in what is called a segmenter stage. After entering segmenter stage the parasites will egress from the red blood cell as many new merozoites, which struggle to find and invade nearby red blood cells and begin the intra-erythrocytic process again. Upon release of the merozoites from the red blood cell, tumor necrosis factor and other cytokines are also released which explain the cyclical symptomology [9].

This asexual life cycle is broken approximately 1% of the time when a ring will form a microgametes or a macrogametes [9]. These sexual stages can be brought into the mosquito during a blood meal and will develop as mentioned above. The complete *Plasmodium spp.* life cycle is depicted in figure 1.

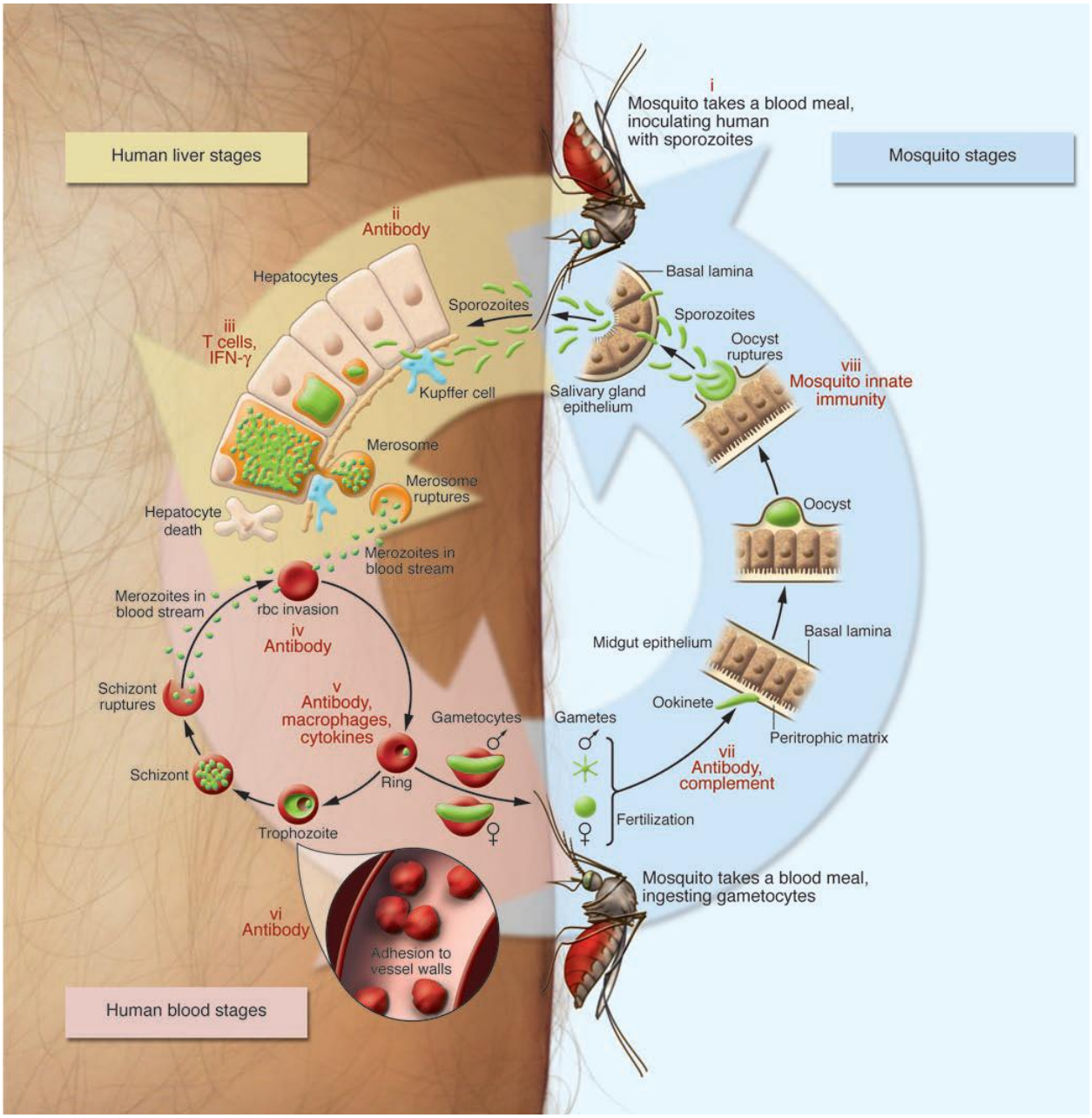


Figure 1 *Plasmodium spp.* Life Cycle [8]

Current Treatment and Prevention Strategies

Per the World Health Organization (WHO) "Malaria is an entirely preventable and treatable disease, provided that currently recommended interventions are properly implemented" [2]

Prevention

"The goals of malaria vector control are two-fold:

1) To protect individual people against infective malaria mosquito bites
2) To reduce the intensity of local malaria transmission at community level by reducing the longevity, density and human-vector contact of the local vector mosquito population" [2]

Insecticide Treated Bed Nets-

The best preventative measure for malaria is utilizing insecticide treated bed nets (ITN's). These nets, originally tested in the 1990's, showed a remarkable reduction in child mortality of over 20% [10]! As early as 1898, the recognition that mosquito nets could reduce the parasite load was being taught by Sir Ronald Ross [11]. The practice of saturating the bed nets in pyrethroids shows similar decreases in child mortality and also shows some proximity protection as well [12]. These long-lasting insecticide treated nets (LLIN) significantly decreased the parasite load and the WHO recommended ceasing distributing a set number per household and start distributing these nets universally as the protection is far greater under a net than it is by proximity only [13]. Also, historically 96% of those who have a bed net will use it, suggesting that this could be a great preventative measure [2].

Indoor Residual Spraying-

A second line of defense is indoor residual spraying (IRS) of insecticides, preferably long-lasting insecticides. This method requires multiple rounds of spraying each year and can be somewhat costly, especially for very-low income countries [14]. Remarkably, 2010 showed an increase in IRS usage with over 70% of countries using long-lasting pyrethroids. Although many countries in Africa reported decreased usage of IRS, the western world (specifically China) more than compensated to bring the increased total above what was done previously. The dominating use of pyrethroids for both ITN's and in IRS promotes concern for possible vector resistance [2]. IRS can be from any of the other three insecticide classes: organochlorine (DDT), organophosphate, or carbamate; whereas pyrethroids are the only ITN approved chemical class at this time. Understandably, cost could be an issue, DDT is roughly the same price as the relatively inexpensive pyrethroids, but organophosphates and carbamates can be as much as 4 times the price of pyrethroids [2]. Overall, of the reporting countries, 11% of at risk populations are currently protected by IRS which, although increasing each year, still needs great improvement, and it is clear that for a global increase in IRS use and availability, funding will need to be increased.

Vaccination-

A third preventative option, an ideal one, could include vaccination against malaria. Since 1910 development of a malaria vaccine has escaped the reach of the scientific community, although many great strides have been taken. Most recently GlaxoSmithKline developed the

RTS,S vaccine which combines circumsporozoite protein antibodies with a hepatitis B vector and the AS01 immune response boosting adjuvant. This vaccine, also known as Mosquirix, is currently undergoing phase III clinical trials. Preliminary findings suggest a decrease in mortality by 50% in infants and children. These findings however, are yet to be verified [15]. If successful, this could be the first malaria vaccine, although improvement is still needed to increase efficacy beyond 50%.

Garcia-Basterio et al.[15] recommend that in order for a vaccine to be effective in preventing malaria it is desirable that it has the following qualities:

-"Be effective preventing clinical disease, severe malaria and transmission in the community

-Be completely safe for young infants and risk populations (pregnant women, people with immune deficiencies or other co-morbidities), with a similar safety profile as other EPI vaccines

-Provide protection against the 5 species of malaria plasmodia

-Provide long-lasting immunity

-Be administrable in the first months of life

-Single oral - dose regime compatible with vaccines of the expanded program on immunization (EPI)

-Easily manufactured, deployed, stored and handled

-Affordable for governments of low income countries

-Stable at room temperature

-Available for travelers of non-endemic areas"

Treatment

Currently there are over seven chemical classes used in the treatment of *Plasmodium spp.* infections including: antifolates, endoperoxides, 4- and 8- aminoquinolines, sulfonamides, amino alcohols, and antibiotics. Of these, the most common currently used chemical classes (aminoquinolines, endoperoxides, and antifolates- including sulfonamides) will be discussed in more detail.

It is important to note that the WHO recommends any treatment of malaria should include combination therapy. As each chemical class has a different mode of action it is believed that the growing trend of drug resistant parasites can be curtailed if combinations of chemicals which inhibit different targets, as well as different stages of life cycle development are used in conjunction. A brief structural representation of the different antimalarial drug classes can be found in figure 2.

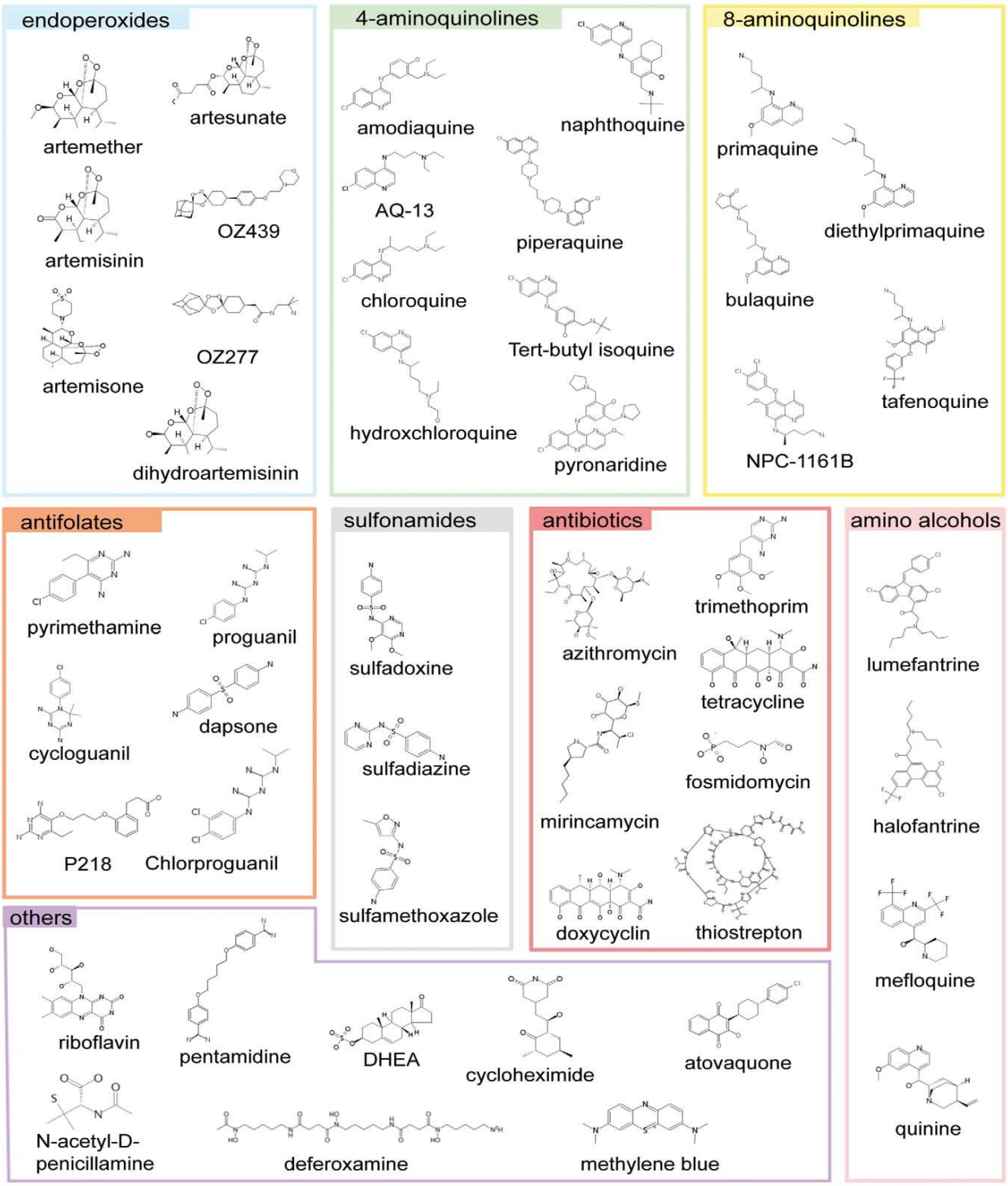


Figure 2 Current Antimalarial Treatment Classes [16]

4- and 8- Aminoquinolines

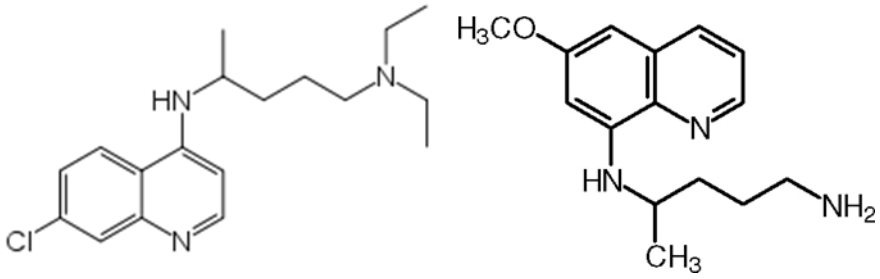


Figure 3- 4- and 8- Aminoquinolines

For almost 400 years aminoquinolines, like those in figure 3, have been used to treat malaria. Isolated from *Cinchona* tree bark in the Andes mountain range, quinine use was taught by the native South and Central American people to Spanish Jesuits in the early 1600's [17]. Because of the limited cultivation and expense of quinine, it became apparent that direct chemical synthesis was needed. Quinine derivatives were successfully synthesized as early as the 1920's and one particular derivative of note, Resochin, was synthesized in 1934 by Hans Andersag. Unfortunately, Resochin was determined to be slightly more toxic than the previous quinine derivative in use: Plasmochin, and the clinical study of Resochin ended prematurely [18]. Nearly a decade later, under the US *Board for the Coordination of Malarial Studies*, Resochin was rediscovered as the substance SN-7619, and was named Chloroquine though it was structurally the identical of Resochin [18].

By the 1960's, Chloroquine became one of the most widely sold of all medications and its ability to serve as both a prophylactic and a preventative treatment only increased its antimalarial value [7]. Chloroquine destroys the parasite by entering into the parasite digestive vacuole and binding hematin (a toxic by-product of hemoglobin digestion by the parasite.).

Normally, the hematin is polymerized into an inert hemozoin crystal. This detoxification is disrupted by chloroquine and the build-up of toxic hematin destroys the parasite [19].

In 1955 the WHO began the Malaria Eradication Program utilizing chloroquine as the sole treatment for infected humans, and the insecticide DDT to tackle the vector control [8]. Using these resources, the WHO successfully eradicated malaria from 24 countries by 1982. Unfortunately, the nations where malaria was not eradicated soon were dominated by a rapid wave of chloroquine resistant *Plasmodium falciparum* [20].

These resistant strains of *P. falciparum* were shown to have mutations in key transport proteins which allowed the parasite to decrease chloroquine concentrations in the digestive vacuole. This transport protein is called *Plasmodium falciparum* chloroquine resistant transporter protein (pfcrt). Because of the decreased efficacy of chloroquine, many nations discontinued its use and switched to antifolate combination therapy. Interestingly, after 8 years of discontinued chloroquine use to treat malaria in Malawi, a test group of 210 patients treated with chloroquine showed that chloroquine sensitivity was restored and that the use of chloroquine was much more effective at reducing host parasitemia than the antifolate combination therapy [21]. Similar studies in other nations also showed increased chloroquine efficacy after discontinued use for some period of time. This lends credibility to the idea that if a chemical treatment is shown to have resistance, it must be temporarily discontinued for some time and then it may be brought back into use. The principle concept behind this is the belief that the mechanism of resistance will be lost from the parasite over time. Jensen et al. describe this practice as “drug cycling”. How effective this method will be over time, and whether or not this method can be applied to other antimalarial drugs remains to be seen [7].

Antifolates and Sulfonamides

Because of a rise in chloroquine resistant parasites, another chemical class of antimalarials needed to be developed. This new chemical class was antifolates. Antifolates inhibit the production of tetrahydrofolate, a necessary step in DNA, RNA, and protein metabolism. Two signature targets of antifolates in *Plasmodium spp.* include inhibition of dihydropteroate synthase (DHPS) by sulfonamides and inhibition of dihydrofolate reducatase (DHFR) by pyrimethamine or cycloguanil. (Figure 4).

DHPS catalyzes the production of 7,8 dihydropteroate from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate and p-Aminobenzoic acid (pABA)[22]. It is clear that sulfonamides, like sulfadoxine, bind to DHPS and, due to structural similarities, inhibit pABA from interacting [23]. This is antagonistically reversible inhibition, as increasing pABA concentrations in the media has been shown to decreases sulfadoxine activity [24].

In addition to sulfonamide mediated DHPS inhibition, another class of antifolates, pyrimethamine, cycloguanil, etc., inhibit dihydrofolate reductase (DHFR). These chemicals have a strong structural resemblance to dihydrofolic acid (the natural substrate of DHFR) and are able to bind to DHFR and inhibit tetrahydrofolate production [25]. One of the major reasons that antifolates, like pyrimethamine, are so advantageous in the treatment of malaria is the fact that protozoan DHFR has a much greater affinity for these inhibitors than human DHFR [26].

Unfortunately, within a short number of years of acceptance as an antimalarial treatment, cases of antifolate drug resistant *Plasmodium* strains surfaced [27]. Most methods of resistance include DHFR or DHPS gene point mutations [28, 29]. which significantly decrease the binding affinity for the drug(s). These mutations could have arisen from the many years of sulfadoxine

and pyrimethamine combination therapy that replaced chloroquine treatment throughout Africa. Although inexpensive and valued for its single dose treatment, the slow excretion of these drugs from the body could also have facilitated resistance development [3].

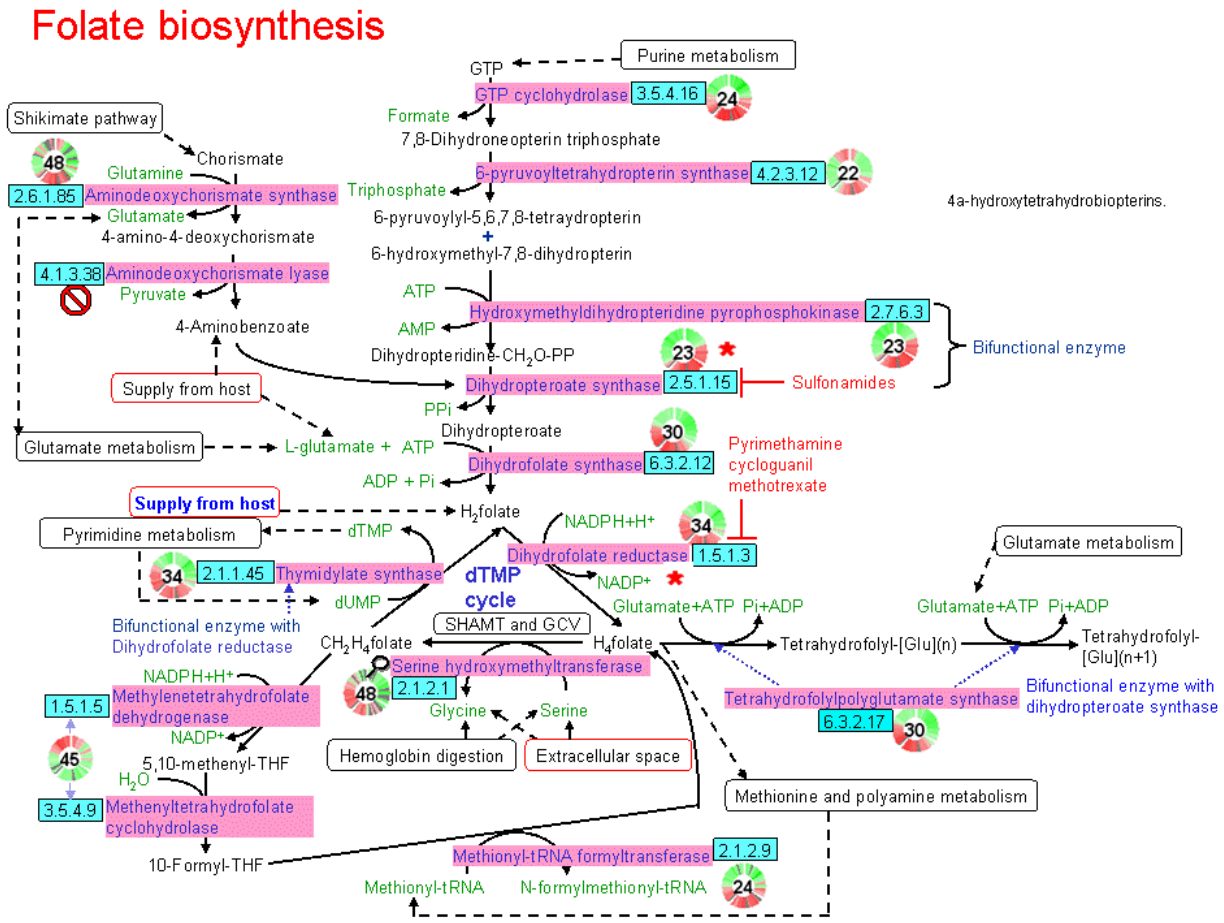


Figure 4- Folate Biosynthesis (http://priweb.cc.huji.ac.il/malaria//maps/folatebiopath.html)

<u>Endoperoxides</u>

Endoperoxides are derived from the parent compound artemisinin (Figure 5). Artemisinin, or qinghaosu, is a natural compound from the plant *Artemisia annua*. Lacking the

typical nitrogen-substituted rings, the sesquiterpene lactone artemisinin has a peroxide bridge making it very unique among antimalarial drug classes [30]. Although the mechanism of action is still unknown, it is believed that within the digestive vacuole the peroxide reacts with the heme to produce free radicals [31] which can lead to the disruption of lipid membranes [32] and the alkylation of specific intracellular targets (heme or other proteins like metalloporphyrins [33]).

One of the great benefits of Artemisinin is a rapid treatment response backed with a short half-life [34]. Most cases of infection can be eliminated completely after the first 48 hours of artemisinin treatment [35]. This swift action also provides successful treatment of potentially fatal cerebral malaria [36]. Unfortunately, the speed of action, like a two edged sword, has been cited in studies as a potential for developing drug resistance, as many patients after the first dose would begin to feel better and would stop treatment before total parasite elimination was completed [37, 38]. In fact, artemisinin treatment failures have already been announced in a few isolated instances. Most of these cases are from regions known to cultivate multi-drug resistant strains and many question if the failure was truly from growing resistance or from the patient's medication non-compliance. In any case, this unusual delay in the parasite development of drug resistance could arise in part from artemisinin's very short half-life as discussed above which could prevent adaptive resistance typically formed in lingering sub-therapeutic drug level environments. Another cause for delayed artemisinin resistance could be the early decision to use artemisinin drugs in combination therapies (ACT's) with longer acting drugs [3]. Numerous studies show that ACT treatment results in decreased rates of recrudescence and cases of latent infection. Some examples of artemisinin deriviates and ACT use are shown in Figure 5.

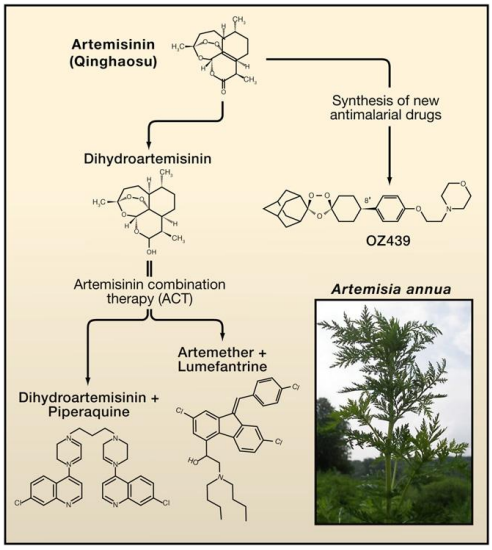


Figure 5- Artemisinin Derivatives and ACT's [39]

Malaria Therapeutics Development

The ever increasing rate of drug resistance has awakened a renewed effort in drug discovery over the past decade. In fact most current treatment options show some degree of resistance or exhibit the classical signs of the emergence of resistance (Table 1)

Table 1- Existing Antimalarial Drugs and Their Use [40]

Common name	Chemical class	Clinical use	Resistance
Artemisinins (artemether, artesunate, dihydroartemisinin)	Sesquiterpene lactone endoperoxide	In artemisinin-based combination therapies (ACTs)	Possibly emerging
Lumefantrine	Arylamino alcohol	Most common first-line antimalarial therapy in Africa, in combination with artemether	No evidence of high-level resistance
Amodiaquine	4-Aminoquinoline	In combination with artesunate in parts of Africa	Limited cross-resistance with chloroquine
Piperaquine	Bisquinoline	In combination with dihydroartemisinin in parts of southeast Asia	Observed in China following single-drug therapy
Mefloquine	4-Methanolquinoline	In combination with artesunate in parts of southeast Asia	Prevalent in southeast Asia
Pyronaridine	Acridine-type Mannich base	Being registered for combined use with artesunate	No cross-resistance with other drugs reported
Quinine/quinidine	4-Methanolquinoline	Mainly for treating severe malaria, often with antibiotics	Exists at a low level
Atovaquone	Naphthoquinone	In combination with proguanil (a biguanide) for treatment or prevention	Has been observed clinically
Chloroquine	4-Aminoquinoline	Former first-line treatment for uncomplicated malaria	Widespread
Pyrimethamine	Diaminopyrimidine	For intermittent preventive treatment, combined with sulphadoxine (a sulphonamide)	Widespread
Primaquine	8-Aminoquinoline	For eliminating liver-stage parasites, including dormant forms of *Plasmodium vivax*	Unknown

The ability to find new antimalarial drugs requires collaborations between pharmaceutical companies, academia and government agencies for any hope of a speedy resolution. Two such collaborations, Guiguemde et al. and Gamo et al., submitted their work in 2010 and showed how partnerships can be used to resupply our antimalarial pipeline [41, 42]. Both studies used high-throughput screening of vast chemical libraries to find new chemical classes that demonstrate antimalarial activity. Medicines for Malaria Venture (MMV) is a drug discovery hub where pharmaceuticals, academia and government funding compile data and collaborate to find the cure for malaria. Unfortunately, even with this collaborative support effort, finding novel antimalarial candidates is proving to be very difficult. The current antimalarial pipeline is shown in Table 2. In order for the pipeline to maintain continuous flow, new leads must frequently be identified. One of the most common methods of lead generation is through screening of synthetic compound or natural product libraries. Once leads are identified, medicinal chemistry can begin with lead optimization. The lead will cycle multiple times between screening and optimizing until the best possible pre-clinical candidate is generated.

Table 2- MMV Antimalarial Pipeline
(http://www.mmv.org/research-development/rd-portfolio.) [43]

Research		Translational			Development		
Lead generation	Lead optimization	Preclinical	Phase I	Phase IIa	Phase IIb/III	Registration	Phase IV
Novartis MP	Novartis (2 projects)	DSM265	GNF156	OZ439	AZCQ		Coartem® *Dispersible*
GSK MP	GSK (2 projects)	Aminoindole		NITD609	Tafenoquine		Artesunate for injection
Broad & Genzyme MP	Sanofi (1 project)	P218 DHFR			Pyramax® Paediatric		Eurartesim®
Open source drug discovery	St.Jude/ Rutgers/USF Antimalarials	ELQ-300			Eurartesim® Paediatric		Pyramax®
Sanofi Orthologue screen	Heterocycles	21A092			Argemone mexicana		ASAQ Winthrop
AstraZeneca MP	Dundee Antimalarials	MMV390048			Nauclea pobeguinii		SP+AQ
Kinases	DHODH						
18 other Projects	Oxaboroles						
	Tetraoxanes						
	Ferrer-GSK (1 Project)						
	21A092 back-up						

Compounds from synthetic libraries have known chemical synthetic schemes and possibly some understanding of mechanism of action from previous screening in other systems. In contrast, natural product libraries are typically extracts containing up to hundreds of compounds which can be screened for activity against various targets/organisms. If the extract shows some activity it will be sent for dereplication, a process that isolates the compounds from

one another within an extract. In either case, synthetic or natural, once a compound is identified as the source of activity, cytotoxic analysis is done to rule out any early cytotoxic concerns and to develop a therapeutic window called the selectivity index. It is also common to run an ADME (Absorption, Distribution, Metabolism, and Excretion) profile on the early lead compound to determine any preliminary pharmacodynamics or pharmacokinetic pitfalls. Following this front-end intensive pattern for drug lead discovery will save millions of dollars and significantly speed up drug approval processing once the drug candidate is found.

Marine Natural Products

With the oceans covering over 70% of the world and 34 of the 36 known phyla of life contained therein, marine life presents itself as a relatively untapped reservoir for novel chemotherapeutics. In the 1950's the first bioactive marine chemicals spongothymidine and spongouridine were purified from the *Tethya crypta* sponge [44]. Since those initial discoveries, over 15,000 novel and bioactive marine natural products have been isolated [45] and many have shown to have excellent anti-inflammatory, antifungal, anti-infective, antimicrobial, anticancer, antituberculosis, and antiprotozoal activity. A few examples of bioactive marine natural products and their targets are listed in table 3. Many of these marine natural products (MNPs) have gone on to clinical trials and many others are still in early development.

In 1992, the sesquiterpenes chemical class was isolated from the Australian sponge *Acanthella klethra* and was shown to have antimalarial activity [46]. Over the following 20 years many different chemical classes showing potent antimalarial activity have been identified from marine life; Including sponges, marine bacteria, and colonial ascidians [47].

Table 3- Selection of marine compounds with potent and varied activities.[48]

Drug Class	Compound	Organism	Chemistry	IC_{50}
Antibacterial	Ascochytatin	Fungus	Polyketide	0.3µg
Antibacterial	L-Amino acid oxidase SSAP	Rockfish	Protein	0.078-0.63 µg/mL
Antibacterial	Arenicin-1	Polychaete	Peptide	2µg/mL
Antibacterial	Isoaaptamine	Sponge	Alkaloid	3.7µg/mL
Antibacterial	Sesterterpenes	Sponge	Terpenoid	1.56-12.5µg/mL
Anticoagulant	Anticoagulant pepetide	Bivalve	Protein	77.9nM
Antifungal	Holothurin B	sea cucumber	Triterpenoid glycoside	1.56µg/mL
Antifungal	Neopeltolide	Sponge	Polyketide	0.62µg/mL
Antiprotozoal	Plakortide	Sponge	Polyketide	0.5-2.3µg/mL
Antiprotozoal	Viridamides A & B	Bacterium	Peptide	1.1-1.5µM
Antiprotozoal	Chaetoxanthone B	Fungus	Polyketide	1.5µg/mL
Antituberculosis	Bipinnapterolide B	Coral	Terpenoid	128 µg/mL
Antituberculosis	8'-O-demethylisonigerone	Fungus	Polyketide	21.5 & 43.0 µM
Antituberculosis	Parguesterols A and B	Sponge	Triterpenoid glycoside	7.8 & 11.2µg/mL
Antiviral	Esculetin ethyl ester	Sponge	Polyketide	46 µM
Antiviral	*Cryptonemia crenulata* galactan	Alga	Polysaccharide	0.8-16µg/mL
Antiviral	6,6'-Bieckol	Alga	Skikimate	1.07-1.72 µM
Antiviral	Mirabamides A, C and D	Sponge	Peptide	0.041-3.9 µM

CHAPTER TWO: MATERIALS AND METHODS

Culturing *P. falciparum*

P. falciparum Dd2 and 3D7 strains were cultured using a modified Trager and Jensen method [49] in RPMI media with L-glutamine (Invitrogen) and supplemented with 25mM HEPES, 26 mM $NaHCO_3$, 2% dextrose, 15mg/L hypoxanthine, 25mg/L gentamycin, and 0.5% Albumax I. Culture media was changed daily and incubated at 37°C in 5% CO_2 and 95% air. The 3D7 strain is a chloroquine sensitive strain characterized as chloroquine sensitive, pyrimethamine sensitive, mefloquine sensitive, and artemisisin sensitive. The Dd2 strain was used as a chloroquine resistant strain with resistance to chloroquine, pyrimethamine, and mefloquine but sensitivitity to artemisinin. A blood smear was done daily and a Giemsa stain was used to determine parasitemia. When parasitemia reached 10-15%, the culture was split down and resupplied with washed and 50% diluted A+ blood received from the Central Florida Blood Bank.

SYBR Green-I Fluorescence Assay

In comparing antimalarial screening assays it is clear that SYBR Green I is both less expensive than PICOGREEN and [H^3]-hypoxanthine incorporation assays and also produces the lowest signal to noise ratio of the three assays in screening of natural product extracts [50] Given these two advantages, SYBR Green I was selected as our means of determining antimalarial activity. Because the *Plasmodium falciparum* host is the RBC which has no DNA, and since SYBR Green is a known DNA intercalating agent which will emit excitation induced light only

after binding to DNA, this assay can be used to quantify DNA levels and therefore, to quantify inhibition levels.

Different dilutions of the compound/fraction in 1 µl of the culture medium were added to 99 µl of *P. falciparum* culture at a 1% parasitemia and 2% hematocrit in 96-well plates. Maximum DMSO concentration in the culture never exceeded 0.125%. Chloroquine at 1 µM was used as a positive control to determine the baseline value. Following 72 hours incubation at 37ºC, the plates were frozen at -80ºC. After thawing the plates at room temperature 100 µl of lysis buffer (with 20mM Tris-HCL, 0.08% Saponin, 5mM EDTA, 0.8%Triton X-100, and 0.01% SYBR Green I) was added to each well. Plates were incubated in the dark for 30 minutes at 37ºC followed by fluorescence emission reading using a Synergy H4 hybrid multimode plate reader (Biotek) set at 485nm excitation and 530nm emission.

Fibroblast Cytotoxicity Assay

Compounds were evaluated for cytotoxicity using NIH/3T3 fibroblast cells. A 384 well plate was seeded with 2,500 cells/well (total volume 50µL) and incubated for 24 hours. Serial dilutions of the compound were added to the plate and plates were incubated for an additional 48 hours. Fifty µL MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), CellTiter 96® Aqueous non-radioactive cell proliferation assay, Promega] reagent was added to each well and the plates were incubated for an additional 3 hours. Cell viability was obtained by measuring the absorbance at 490nm using Synergy H4 hybrid multimode plate reader (Biotek).

Sensitivity and Reliability Statistical Analysis

In order to validate active hits and recognize and quantify sensitivity and accuracy of each assay we utilized a Z-factor analysis. This assay was originally developed by Zhang et al [51], for HTS validation. The equation is shown below. The Z-factor is a range from 0 to 1. The closer the score is to the value of 1, the more ideal are the assay conditions and the more valid are the hit results. A value below 0.5 is considered unreliable and perhaps requires some modifications before the assay can be re-run. A value between 0.5 and 1 is considered acceptable for hit validation

$$\boldsymbol{Z - factor = 1 \pm 3\left(\frac{(\sigma p - \sigma n)}{(\mu p - \mu n)}\right)}$$

Where σp represents the standard deviation of the positive controls and σn is the standard deviation of negative controls. Also, μp represents the mean of the positive controls and μn represents the mean of the negative controls.

CHAPTER THREE: IDENTIFICATION OF NOVEL ANTIMALARIALS FROM MARINE NATURAL PRODUCTS

Primary Screening of HBOI Peak Fractions

The Harbor Branch Oceanographic Institute in Fort Pierce, Florida began marine natural product drug discovery in 1984. The HBOI collection program has two specific aims: to maximize taxonomic diversity; and to evaluate how ecological factors relate to secondary metabolite expression. Dedication to these two primary aims has already resulted in the publication of over 100 bioactive marine natural products and over 96 patents to protect these discoveries. Their vast library includes many uncommon marine organisms, primarily collected by the Johnson Sea-Link submersible, which is capable of collecting samples at depths of almost 1000m, an order of magnitude deeper than typical scuba-access derived collection (<100m).

Given the proximity of HBOI and their unique collection of marine life fractions we began a collaborative effort to screen over 2,600 marine natural product peak fractions in search of novel antimalarial chemical scaffolds. This joint UCF-HBOI drug development scheme is depicted in figure 6 below

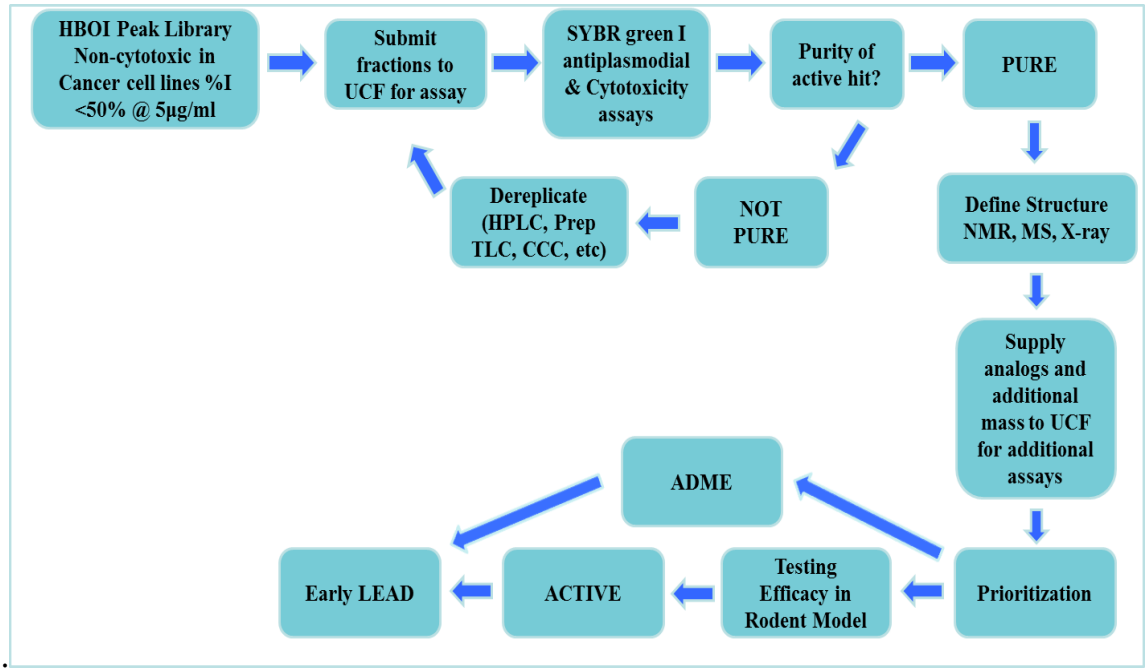


Figure 6-UCF-HBOI Drug Development Scheme

In the screening process we quickly identified two-hundred fifty-three active fractions against the chloroquine sensitive *Plasmodium* 3D7 strain. Any fraction which demonstrated greater than 70% inhibition of parasite at a concentration of 5µg/ml was defined as an active fraction. A list of macroorganims sources for these fractions can be found in table 4.

Table 4 Macroorganism Sources of Active Peak Fractions

Active Peak Fraction Origins (active = >70% inhibition @ 5µ/ml)				
	Coral	**Sponge**	**Mollusk**	**Other**
Number of Different Species with Active Fractions	3	13	1	3

The active fractions were next prioritized by selecting the top 2 active fractions per species. This resulted in 31 active fractions which were then screened against the 3d7 parasite

strain at different concentrations to determine the IC_{50}. Table 5 represents the result of this screening process. These results were sent back to HBOI where the fractions were further purified and dereplicated.

Table 5 Antimalarial Activity of HBOI Peak Fractions

Fraction	3D7 IC_{50} (ug/ml)	Fraction	3D7 IC_{50} (ug/ml)	Fraction	3D7 IC_{50} (ug/ml)
12.A09	0.73	18.F01	0.98	30.A01	0.99
13.H07	1.1	18.G07	0.44	31.C08	1.1
16.A01	0.67	22.A01	0.82	32.C02	0.08
18.A01	0.62	23.F01	1.1	33.A03	1.2
18.A10	0.52	24.C05	0.21	39.A05	0.75
18.C05	0.23	27.B10	0.51	39.A06	0.6
18.D08	0.73	27.C10	0.47	39.A10	0.85
18.EO3	0.96	29.A07	0.52		

All data are the mean of at least three independent experiments.

Primary Screening of HBOI Purified Fractions

As the prioritized active fractions were purified and dereplicated, they were fractionated out and sent back to UCF for additional screening. Over 200 purified fractions were received from HBOI in two separate shipments. The purified fractions from the first shipment were screened against the chloroquine resistant Dd2 strain and the results are shown in table 6.

Table 6- Purified fractions antiplasmodial IC_{50}

Fraction	Field ID	Dd2 IC_{50}	Fraction	Field ID	Dd2 IC_{50}
4.B07	Aplysina sp.	1.20	18.D08	Paramuriceidae	0.73
7.G05	Peysonellia	1.30	18.EO3	Paragorgai	0.96
7.G11	Peysonellia	1.00	18.F01	Spongosorites	0.34
9.G07	Axinellida?	0.80	18.G03	Paramuriceidae Unid. sp.	0.83
10.C11	Spirastrella sp.	0.90	18.G07	Paramuricea sp.	0.44
10.D08	Gastropoda, Aplysia? sp.	0.10	22.A01	Dendroceratida	0.82
10.D09	Gastropoda, Aplysia? sp.	0.50	23.F01	Erylus sp.	1.12
11.A08	Gorgonacea	1.20	24.C05	Pachastrellidae	0.46
11.H01	Axinellida	1.20	27.C10	Amphibleptula	0.47
11.H02	Axinellida	1.20	29.A07	Axinellidae	1.24
12.A09	Axinellida	0.74	30.A01	Spongiidae	0.99
12.A10	Axinellida	0.26	30.B11	Spongiidae	1.43
13.H07	Leiodermatium? sp.	1.13	31.B11	Choristida	1.40
15.D04	Xestospongia? sp.	1.58	31.C08	Choristida	1.17
15.D07	Xestospongia? sp.	1.30	32.C02	Halichondriidae	1.20
15.H06	Halichondrida?	1.25	33.A03	Spongiidae	1.20
18.A01	Poecillastra	1.93	39.A05	Aplysina	0.75
18.A10	Plexauridae	1.44	39.A06	Aplysina	0.60
18.C04	Myrmekioderma styx	0.65	39.A10	Aplysina	0.85

All data are the mean of at least three independent experiments.

All fractions with an IC_{50} at or below 1µg/mL were further screened for cytotoxicity. Unfortunately, several of these fractions were depleted prior to cytotoxicity screening and were hence removed from further analysis at this time. The resulting selectivity indices are shown in table 7 and further dereplication of a few of these fractions was included in the second shipment from HBOI. The dereplication of the remainder of these active fractions will be occurring in the near future.

Table 7- Selectivity indices of all fractions with <1µg/mL IC_{50} in Dd2

Fraction	Field ID	Dd2 IC_{50}	NIH 3T3 IC_{50}	Selectivity Index
7.G11	Peysonellia	1.00	47	47
9.G07	Axinellida?	0.80	33	41
10.C11	Spirastrella sp.	0.90	48	53
10.D08	Gastropoda, aplysia? sp.	0.10	31	310
10.D09	Gastropoda, aplysia? sp.	0.50	29	59
18.D08	Paramuriceidae	0.73	>50	>68
18.EO3	Paragorgai	0.96	>50	>52
18.G07	Paramuricea sp.	0.44	>50	>113
27.C10	Amphibleptula	0.47	>50	>105
39.A05	Aplysina	0.75	>50	>67
39.A06	Aplysina	0.60	>50	>83
39.A10	Aplysina	0.85	>50	>59

All data are the mean of at least three independent experiments.

The second shipment of purified fractions also contained several active purified fractions from 7 distinct species which all showed to have antiplasmodial activity in the initial screening tests. Among this shipment were the purified fractions from species which had multiple active fractions in the initial screening, including: *Choristida sp.*, *Bebryce sp.*, *Amphibleptula sp.*, *Aplysina sp.*, *and Auletta sp.* derive fractions. The top fraction from each organism is listed in table 8. These results have been sent back to HBOI and further dereplication and purified compound isolation from all active purified fractions is underway.

Table 8- Potent antimalarial HBOI purified fractions

Organism ID	Genus	IC_{50} (µg/mL)
10-V-00-1-004	*Bebryce sp.*	0.47
31-III-89-2-003	*Amphibleptula sp.*	0.73
10-V-00-3-009	*Aplysina sp.*	0.77
11-V-00-3-009	*Auletta sp.*	0.83
23-XI-96-1-006	*Choristida sp.*	1.2

All data are the mean of at least three independent experiments.

CHAPTER FOUR: STRUCTURE ACTIVITY RELATIONSHIP PROFILE FOR PREVIOUSLY IDENTIFIED HIT- NORTOPSENTIN A

Primary Screening of Nortopsentin A Analogs

Early screening of the first 336 HBOI purified fractions resulted in a potential hit from the genus *Spongosorites* spp known as Nortopsentin A. The bis(indoyl)imidazole Nortopsentin A (Figure 8).

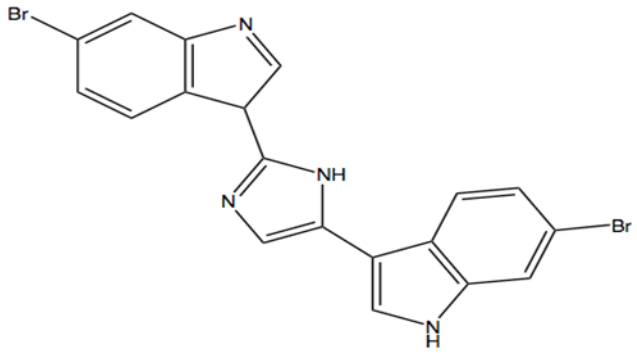


Figure 7 Nortopsentin A Structure

The antimalarial activity of nortopsentin A had never been shown before. Nortopsentin A was purified and sent to UCF for additional screening. Nortopsentin A demonstrated effective antimalarial activity in both the 3D7 (choloroquine sensitive) and the Dd2 (chloroquine resistant) strains.

The mechanism of action for nortopsentin A was previously studied in our laboratory by culturing parasites in nortopsentin A drug treated culture. In that study nortopsentin A was shown to inhibit development beyond the early trophozoite stage which supported the claim of previous studies which showed that topsentin compounds can intercalate with DNA and inhibit DNA synthesis [52].

With this potentially novel mechanism of action among antimalarials, nortopsentin A was escalated in lead development. Recent screening for antiplasmodial and cytotoxicity IC_{50} show an IC_{50} for Nortopsentin A against Dd2 at 580nM and a cytotoxicity IC_{50} value of 6µM as shown in Figure 9.

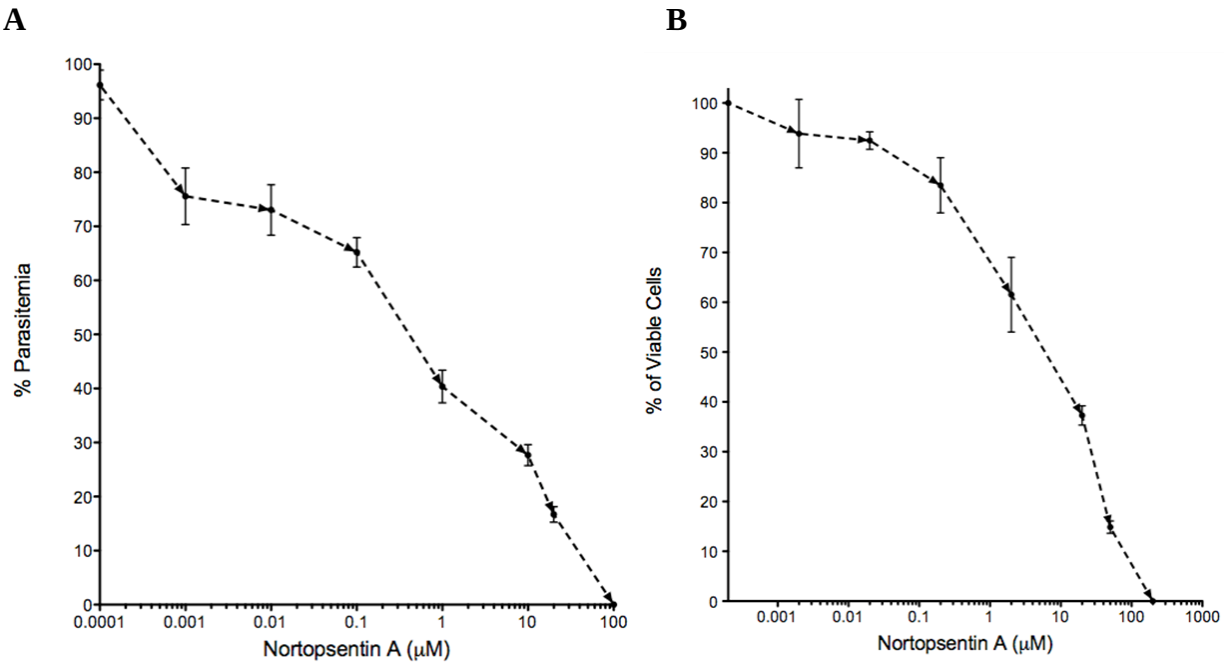
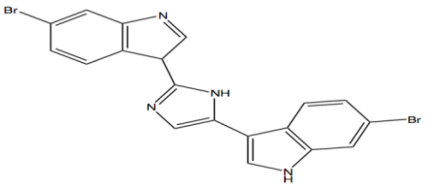
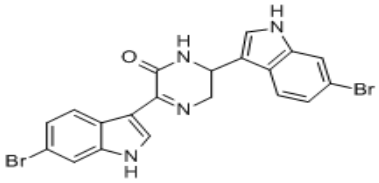
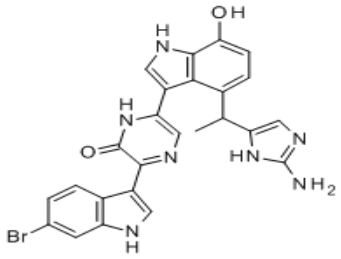
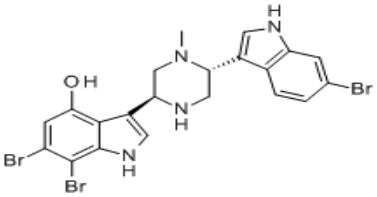
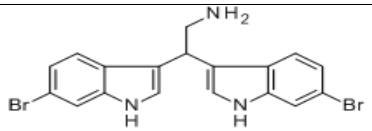
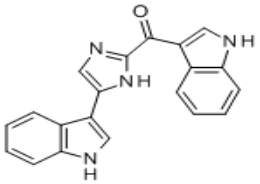
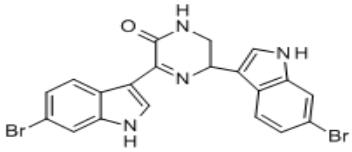


Figure 8- In-Vitro Antimalarial and Cytotoxic Analysis of Nortopsentin A.
(A) Determination of the antimalarial activity of nortopsentin A in Dd2 strains of *P. falciparum*. Asynchronous cultures were exposed to different concentrations of inhibitor for 72 hrs. (B) Cytotoxicity of nortopsentin A. Varying concentrations of nortopsentin A was incubated with NIH 3T3 fibroblast cells for 48 hours to determine the cytotoxicity IC_{50} value. All data shown are the result of at least 3 independent experiments.

Structure Activity Relationship Profiling

In order to assist medicinal chemistry in lead optimization, it is important to develop a structure activity relationship for any potential lead. For the compound nortopsentin A, six chemical analogs were selected. These six analogs, along with nortopsentin A, were screened against the chloroquine resistant *Plasmodium* strain Dd2. Following an IC_{50} determination, all compounds were also screened for cytotoxicity against the NIH 3T3 fibroblast cell line using the MTS cell viability assay described in the methods section. This data was collected and used to determine a selectivity index for each compound and is shown in Table 8.

Table 9 Bis(indolyl)imidazole Structural Analogs

Compound Name	Structure	DD2 EC50 (μM)	Cytotoxicity NIH 3T3 (μM)	Selectivity Index
Nortopsentin A		0.58	6	10
Hamacanthin-A		3.2	30	9.4
Dragmacidin-D		5.2	27	5.2
Dragmacidin		6.4	7	1.1
Bis(2,2)-6-Br-indol-3yl-Ethyl-amine		6.5	11	1.7
Deoxytopsentin		8.4	20	2.4
Hamacanthin-B		>20	38	<1.9

All data are the mean of at least three independent experiments.

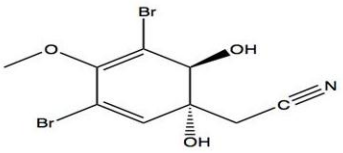
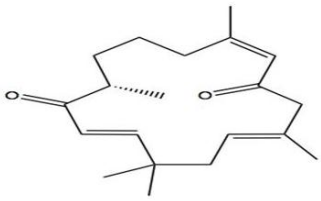
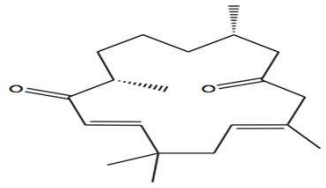
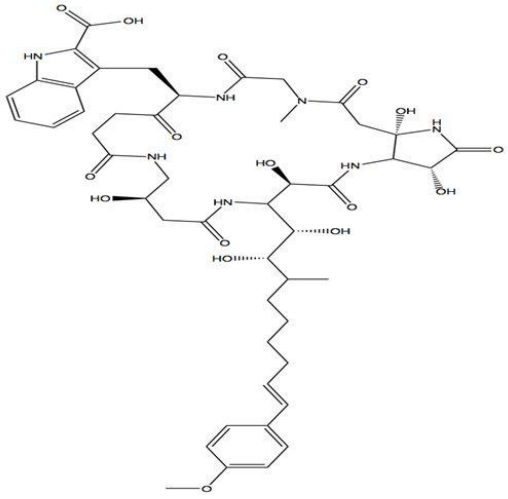
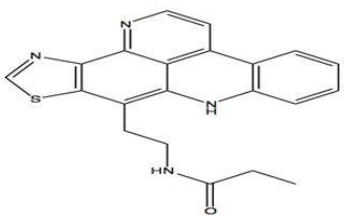
CHAPTER FIVE: DISCUSSION

HBOI Marine Peak Fraction Library

Culture screening of over 2,600 marine peak fractions from the Harbor Branch Oceanographic Institute against the 3d7 strain of *Plasmodium falciparum* resulted in 253 fractions which inhibit at least 70% of parasite growth at 5µg/mL. This nearly 10% hit rate is consistent with typical natural product library screening showing significantly higher hit rate percentages as compared to synthetic library screening, as mentioned previously. From these 253 compounds we identified 20 species of marine organisms which inhibit *Plasmodium falciparum* growth from which the 35 fractions were selected for further study. 83% of the 35 fractions were also active in the chloroquine resistant *Plasmodium falciparum* Dd2 strain.

Further dereplication led to purified fractions from 6 different species, one of which, *Spongosorites sp*, is the source of the antimalarial near-early lead compound nortopsentin A as discussed previously. Five additional compounds from four chemical classes have also been identified from these species and they are shown along with their genus source in table 10. Additional structure activity relationship profiling, pharmacodynamics/pharmacokinetic studies and medicinal chemistry for these five compounds may reveal additional novel antimalarial early drug leads.

Table 10-UCF-HBOI novel antimalarial compounds

Compound Structure	Source of compound (Genus)
	*Aplysina sp.*
	*Bebryce sp.*
	*Bebryce sp.*
	*Amphibleptula sp.*
	*Choristida sp.*

Nortopsentin A Structure Activity Relationship

The preliminary screening of nortopsentin A showed antimalarial activity in both chloroquine sensitive and chloroquine resistant strains. A novel mechanism of action among antimalarial drugs was also demonstrated. To further escalate nortopsentin A towards lead development, a collection of six structural analogs, along with nortopsentin A, was sent from the HBOI library for development of a structure activity relationship.

Of all the compounds tested, nortopsentin A was the most potent and had the greatest selectivity. Addition of a keto group both decreased antiplasmodial activity and selectivity as seen in deoxytopsentin. With respect to all the nitrogenated six member heterocycle rings tested, low µM antiplasmodial activity was maintained and selectivity decreased steadily with respect to the level of saturation which could be due to subsequent molecular loss of planarity. The only exception to this trend was hamacanthin A. Interestingly, when the pyrazinone linker attachment in hamacanthin A changes to the 5 position the antiplasmodial activity is lost, as seen with hamacanthin B. It seems from this structure activity relationship profile that molecular planarity and a maintained distance between the two indole rings is essential for optimal antimalarial activity.

Although nortopsentin A had the best activity and selectivity, further medicinal chemistry is needed to widen the selectivity index before the compound can be escalated to early drug lead status.

Future Directions

There is a critical need to find new antimalarial chemical scaffolds. This screening of 2,835 marine peak fractions has identified 5 new chemical classes which inhibit *Plasmodium falciparum* at submicromolar levels. One of these classes, the topsentin class from the *Sporosorites sp.*, appears to intercalate parasite DNA and prevent DNA synthesis. This is a novel mechanism of action amongst current antimalarial drugs. From the structure activity relationship profile it is clear that the antimalarial potency of topsentin compounds is dependent upon the distance between the two indole rings and the molecular planarity of the compound.

Future studies will include medicinal chemistry optimization of nortopsentin A to enlarge the selectivity index before the compound can be escalated in pre-clinical drug development. The mechanism of action and structure activity relationships for each of the four additionally identified scaffolds is also necessary. Furthermore, it is anticipated that dereplication of the purified peak fractions from the nine newly identified species with submicrogram/mL IC_{50}, will likewise reveal additional novel antimalarial scaffolds which can then be escalated along the established UCF-HBOI drug development scheme.

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