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Determination of selectivity and potential for drug resistance of novel antimalarial compounds from nature-inspired synthetic libraries

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DETERMINATION OF SELECTIVITY AND POTENTIAL FOR DRUG RESISTANCE OF NOVEL ANTIMALARIAL COMPOUNDS FROM NATURE-INSPIRED SYNTHETIC LIBRARIES

by

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ABSTRACT

As malaria, caused by *Plasmodium* spp., continues to afflict millions of people worldwide, there is a dire need for the discovery of novel, inexpensive antimalarial drugs. Although there are effective drugs on the market, the consistent development of drug resistant species has decreased their efficacy, further emphasizing that novel therapeutic measures are urgently needed. Natural products provide the most diverse reservoir for the discovery of unique chemical scaffolds with the potential to effectively combat malarial infections, but, due to their complex structures, they often pose extreme challenges to medicinal chemists during pharmacokinetic optimization. In our laboratory we have performed unbiased, cell-based assays of numerous synthetic compounds from chemical libraries enriched with nature-like elements. This screening has led to the discovery of many original chemical scaffolds with promising antimalarial properties. In an attempt to further characterize these scaffolds, the most promising compounds were assayed in order to determine their cytotoxic effects on mammalian cells. In addition, the development of a drug resistant parasite line of *Plasmodium falciparum* to the most promising compound was done in order to determine the relative probability for parasite resistance development.

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CHAPTER ONE: INTRODUCTION

According to the most recent statistics presented by the World Health Organization (WHO) in their annual World Malaria Report 2011, an estimated 3.3 billion people, or roughly half of the global population, are living in areas which are considered at risk of malaria transmission, and, of the 216 million confirmed reports of malaria infection, approximately 655,000 proved to be fatal (WHO 2011). Additionally, 86% of the 655,000 deaths that were reported in 2010 occurred in children under 5 years of age (WHO 2011). The majority (~95%) of these infections and subsequent fatalities occurred in the poverty-stricken countries of Africa and Southeast Asia due to inadequacies in disease prevention, surveillance and treatment within the given countries' healthcare systems (Snow, Guerra et al. 2005). These grim statistics become even more troublesome when one considers that the data for these estimates comes strictly from those cases which were formally documented and reported through national malaria control programs, and, thus, the actual prevalence of clinical episodes and fatalities may be up to 50% higher than the original estimates (Snow, Guerra et al. 2005). The most effective remedy for such dire statistics is the discovery of novel, inexpensive and effective antimalarial chemotherapies.

An Overview of the Malaria Parasite: *Plasmodium spp.*

Plasmodium spp. is the causative agent of one of the most infamous infectious diseases to have ever plagued mankind, malaria. It is classified as a unicellular eukaryotic apicomplexan, and, although there are many different species of *Plasmodium* which afflict humans, including *P. vivax, P. ovale, P. malariae* and *P. knowlesi*, the most burdensome has proven to be *P. falciparum*. *P. falciparum* is by far the most virulent of all the species known to afflict humans, and it alone is responsible for approximately 85% of global malaria fatalities, roughly 600,000 per year (Rich, Leendertz et al. 2009).

The *Plasmodium* Life Cycle

Before discovering a truly novel and effective antimalarial, the parasite's extremely complex life cycle must first be examined and understood. The life cycle involves multiple stages which occur in multiple hosts, but it ultimately begins with the infection of its primary vector, female mosquitos of the genus *Anopheles.* Once the female mosquito obtains a blood meal from an infected host, *Plasmodium* sexual stages (gametocytes) that are comprised of the highly motile microgametocyte (male) and the nonmotile macrogametocyte (female) are ingested, and they subsequently fuse to form a mature ookinete which is the parasite's diploid zygote (Matuschewski 2006). The mature ookinetes then go on to invade the basal lamina of the mosquito's midgut cells where they implant and begin to continue to develop into oocysts. These oocysts undergo multiple mitotic divisions to form sporoblasts and, ultimately, sporozoites

(Matuschewski 2006). Sporozoites free themselves from the oocyst and invade the mosquito's salivary gland where they reside and await inoculation into a human host. This particular part of the life cycle is collectively referred to as the sporogonic cycle.

The life cycle continues once the infected female *Anopheles* mosquito takes a blood meal from a human host. Upon obtaining the blood meal, the sporozoites within the mosquito's salivary glands are inoculated into the underlying blood capillaries. Eventually, the sporozoites are carried within the circulation to the liver sinusoids. The liver sinusoids contain cells known as Kupffer cells which are resident macrophages, and these cells interact with the sporozoites to effectively serve as a bridge between the sinusoid lumen and the liver hepatocytes (Frevert 2004). The sporozoite within the hepatocyte then begins to undergo a form of schizogony via multiple DNA replications to produce a cluster of thousands of merozoites collectively called a merosome (or schizont) (Meis and Verhave 1988). These merosomes have been shown to protect the inner merozoites from phagocytosis, and eventually they are expelled from the host hepatocyte back into the lumens of the liver sinusoids (Sturm, Amino et al. 2006). After a short time, the merosome ruptures and the merozoites are released into the bloodstream to infect the host's erythrocytes. This portion of the cell cycle is collectively called the exoerythrocytic stages, or more simply liver stages, of the parasite.

The newly freed merozoites within the bloodstream mark the beginning of the next phase of the cell cycle known as the erythrocytic phase. First, the merozoite enters the host erythrocyte

and begins its transition to the first stage of this portion of the cell cycle which is known as ring stage for its resemblance to a ring when stained and viewed microscopically. Growth continues on from the ring stage to the second stage known as the trophozoite. During this stage, the trophozoite actively takes up the erythrocyte cytoplasm and utilizes the hemoglobin it obtains as a source of amino acids (Perlmann and Troye-Blomberg 2002). Additionally, the trophozoite stage is where DNA replication begins in order to produce more merozoites. The trophozoite stage begins to transition to the final schizont stage when schizogony is initiated. Similar to the schizont formed during the exo-erythrocytic stages within the hepatocyte, the multinucleated erythrocytic schizont is an aggregate of numerous merozoites. It is formed via multiple rounds of DNA replication with no accompanying cytokinesis, and is a stage in the parasite's life cycle which is unique among all other eukaryotes. Eventually, the schizont, along with the erythrocyte, ruptures and releases the merozoites into the bloodstream to infect even more erythrocytes. This stage of the infection is ultimately when most symptoms become empirically noticeable, and it is the primary stage of the parasites life cycle that is targeted by past and present antimalarial chemotherapies.

Present State of Malaria Control and Prevention Efforts

The first moderately effective effort to globally eradicate malaria came in the 1950s when the World Health Organization (WHO) introduced a program called the Global Malaria Eradication Program (WHO 1999). Through this effort, chloroquine was used extensively in

order to treat infections due to its unprecedented efficacy at the time. In addition, the now banned insecticide known as DDT (dichlorodiphenyltrichloroethane) was used in order to control the *Anopheles* population (WHO 1999). However, the program ultimately ended in the 1970s due to the emergence of drug resistant parasites and *Anopheles* vectors. More recently, in 1999 an effort known as the Medicines for Malaria Venture (MMV) was created. As its name implies, this movement aims to discover and develop novel inexpensive antimalarial therapeutics in order to help the impoverished and most afflicted nations of Africa and Southeast Asia. To date, the MMV has been responsible for the development and delivery of antimalarials to many of these countries. However, the organization's insufficient funding seems to limit their ability to help on a worldwide scale.

Other efforts at malaria prevention include the recently developed RTS,S/AS01 vaccine by GlaxoSmithKline (GSK). This particular attempt at an effective vaccine mimics the parasite's circumsporozoite protein that bears the highest concentration of all surface antigens on the sporozoite (Stout, Slaoui et al. 1997). Although the vaccine has yet to be clinically approved, it recently completed the first stages of Phase III clinical trials in January 2011. Through these trials, two age groups (6-12 weeks and 5-17 months) were given the three doses that comprise the complete vaccination over a three-month time period (one dose each month). However, only the results for the older age group have been presented because only this age group has completed the required 12-month follow-up (Agnandji, Lell et al. 2011). Nonetheless, the

reported vaccine efficacy against clinical malaria cases in this age group was 55.1%, and the efficacy against severe malaria cases was reported to be 34.8% (Agnandji, Lell et al. 2011). Despite the fact the results are not complete due to a lack of results from the younger age group, it is clear that the vaccination is not as effective as one would hope. While the vaccine undoubtedly has the ability to save many lives and much suffering, considering the time it will take to get out of clinical trials and the modest results demonstrated thus far, other developments to prevent and treat malaria are still dramatically needed.

Vector control is yet another means of preventing the spread and prevalence of malaria infection. The most common form of vector control is the use of insecticide-treated bed nets; the majority of which are currently being distributed at no charge in 82 countries throughout the world (WHO 2011). However, some of these bed nets have issues with longevity of use, and others have seen the development of vector resistance to certain insecticides (WHO 2011). In addition to insecticide-treated bed nets, indoor residual spraying of certain insecticides (including the infamous DDT) has also been employed in order to control the *Anopheles* population. However, unlike the insecticide bed nets, indoor residual spraying is much less prevalent throughout the most afflicted nations in the world due to the expense of the insecticides and the lack of capital by the consumer.

With all other control efforts seeing such limited success, it becomes more obvious than ever that there is a dire need for the discovery and development of novel antimalarial chemotherapies.

Current Antimalarial Chemotherapies: Efficacy and Resistance Development

To date, the most effective prevention and treatment method for malaria infection has proven to be the discovery and development of novel compounds which display potent antimalarial activity. In fact, there have been multiple antimalarial compound discoveries within various chemical families since the onset of antimalarial drug discovery research. The most common and widely used antimalarial chemical families include the quinolines, antifolates and artemisinins.

The quinoline family of antimalarial compounds possesses some of the most effective compounds to date, including quinine, chloroquine, mefloquine and primaquine. Quinine should be noted before all others when discussing the quinoline family because it is the parent compound from which all the others were eventually derived (Ridley 2002). Quinine was originally extracted from the bark of the *Cinchona* tree in the 1930s, but, due to its rather long regimen time, which requires three administrations per day for a week or more, the search for more potent structural analogs began (Meshnick 1998; Ridley 2002). Perhaps the most overall effective drug to be developed thus far is chloroquine (CQ), a 4-aminoquinoline, which dominated antimalarial treatments beginning in the late 1930s (Hartl 2004). Ultimately, CQ

works by interfering with the biocrystallization process of heme within the parasite's digestive vacuole that leads to a toxic buildup of heme, ultimately sealing the fate of the parasite (Wellems and Plowe 2001). In fact, this mechanism of inhibition has also been shown to be possessed by other quinolines such as quinine, mefloquine and primaquine (Dom, Vippagunta et al. 1998). CQ was used almost exclusively for 20 years because of its unmatched efficacy; however due to resistance development emerging first in the 1950s in both South America and Southeast Asia, followed by Africa in the 1970s, alternatives to CQ became a necessity (Wellems 2002). CQ resistance specifically is generated via multiple point mutations in the parasite's PfCRT (*P. falciparum* CQ resistance transporter) gene whereby the parasite acquires the ability to pump out CQ to restore the parasite's heme detoxification process (Fidock, Nomura et al. 2000). Another commonly used quinoline is mefloquine (MQ). MQ was proven effective in parasites which are CQ resistant, but studies have shown that additional resistance to it develops extremely fast (Wellems 2001; Price, Uhlemann et al. 2004). Additionally, primaquine has been proven effective, but, if it is not used in conjunction with other antimalarials such as CQ or MQ, it is minimally effective due primarily to an extremely large relapse rate (Baird and Hoffman 2004). Although the quinoline family of antimalarials has historically demonstrated great success in the past, resistance development has been reported to each and every one, so the need for even more effective drugs lingers on.

A second class of antimalarial compounds is collectively known as the antifolates. Antifolates are usually administered in fixed ratio combinations of their two distinct classes: the dihydropteroate synthase (DHPS) inhibitors and the dihydrofolate reductase (DHFR) inhibitors (also called class I and class II antifolates, respectively) (Winstanley and Ward 2006; Nzila 2006). Antifolates are used in combination of the two subclasses because together they have a synergistic effect by inhibiting two separate parts of the folate pathway; this fact also helps slow the emergence of resistance (Ridley 2002). In accordance with their name, these drugs act by inhibiting their respective enzymes, and this inhibition ultimately leads to the interference of DNA synthesis via the depletion of essential cofactors (Winstanley and Ward 2006). Though there are many combinations of antifolates used, the most common is pyrimethamine-sulphadoxine; pyrimethamine represents the DHFR-inhibitor portion while sulphadoxine acts as the DHPS-inhibitor. Originally, this combination showed a greater efficacy than that of CQ, but resistance has been routinely reported since their introduction in the most afflicted countries (such as those in Africa and Southeast Asia) where resistance is much more prevalent (Wellems 2001). Similar to the antimalarials which came before them, resistance to both has been reported to occur due to multiple mutations within specific genes (the *dhfr* gene for pyrimethamine and the *dhps* domain of the *dhps-pppk* gene for sulphadoxine) (Nzila 2006).

The present day gold standard for *Plasmodium* infection lies within the family of compounds known as the artemisinins. The artemisinin family is based on the compound

artemisinin itself, which is derived from a naturally-occurring variety of wormwood known as *Artemisia annua* (or qinghao) (Price 2000). Its original discovery and potency demonstration against malaria in the 1970s immediately led to the development of its near equally potent semisynthetic derivatives, including artesunate and artemether (Dondorp, Yeung et al. 2010). Although the exact mechanism of antimalarial action of the artemisinins is still being debated, it has undoubtedly been shown to affect both the erythrocytic stages and the gametocytes of the parasite in such a way which has never been seen before (Olliaro 2001). However much like every antimalarial which came before them, the artemisinins have their disadvantages. The primary drawback to using artemisinin or one of its derivatives exclusively is their extremely short half-life in comparison to previous antimalarials; regardless of whether one of the semisynthetic derivatives is used or artemisinin itself, all are rapidly metabolized to the primary effective agent known as dihydroartemisinin (DHA). However, DHA also has an extremely short half-life of about one hour (Ridley 2002; Dondorp 2010). This problem ultimately leads to an undesirable extended treatment plan that historically has shown poor patient compliance (RBM/WHO 2001). To remedy this problem, drugs from this family of compounds are given in combination with other known antimalarials that have a much longer half-life (Price and Douglas 2009). This combonatorial treatment plan has been deemed artemisinin combination therapy (or ACT), and it is currently the first line of defense against most *Plasmodium* infections. The most common derivative used is artesunate, and it is frequently paired with either CQ, amodiaquine

(another 4-aminoquinoline related to CQ), MQ or SP (RBM/WHO 2001). Despite the ACTs unprecedented initial success, decreased sensitivity (parasite resistance) to ART and its derivatives was discovered in Cambodia starting in 2004 (Dondorp, Nosten et al. 2009). The exact mechanism of resistance is currently still being studied, but current evidence suggests multiple mutations in multiple genes such as *PfMDR1, PfCRT* and *PfSERCA* (sarcoendoplasmic reticulum calcium ATPase-6) (Dondorp, Nosten et al. 2009). However, this resistance has been reported to be incomplete because worldwide treatment success rates remain, on average, above 90% (Dondorp, Yeung et al. 2010). Nevertheless, resistance is indeed emerging to this class of drugs just like all others previously, so the need for the discovery and development of even more drugs is still a primary goal.

Nature-inspired Antimalarial Therapeutics

Nature has played a key role in antimalarial drug discovery since the very beginning. One of the first antimalarials ever discovered, quinine, was extracted from the bark of the *Cinchona* tree. Additionally, the most potent antimalarial to date, artemisinin, was also extracted from a natural source. However, these compounds were just the leads that set the stage for the evolution of entire classes of antimalarials. After each was discovered, they were subsequently modified using medicinal chemistry techniques to develop multiple semisynthetic compounds that displayed even higher parasite potencies (Kaur, Jain et al. 2009). However, quinine and artemisinin are only the most notable examples; many other lead compounds that display extremely potent antimalarial properties have been discovered from many other natural sources,

such as other terrestrial and marine extracts (Kaur, Jain et al. 2009). History has proven that nature possesses the remedy for problems of its own creation; the only obstacles in the way of development of those remedies are lack of interest in research and development by the pharmaceutical industry as well as undesirable preliminary chemical properties.

One of the pharmaceutical industries' key qualms against the screening of natural products for antimalarial activity is that a discovery could be highly reproducible, thereby preventing patent attainment (Li and Vederas 2009). However, as past antimalarial families have shown, the initial discovery is just the beginning. Often times the crude extract, such as quinine for the quinolines and ART for the artemisinins, only represents a parent compound or scaffold from which other semisynthetic derivatives with even greater efficacies can be generated. These derivatives can then, in turn, be patented and marketed at the pharmaceutical companies leisure. Nevertheless, their lack of interest lingers on, and, until this changes, it will remain exponentially harder to efficiently develop new antimalarials of the nature-inspired genre.

Additionally, many compounds that are isolated from natural sources display undesirable qualities including cytotoxicity, inadequate bioavailability and poor chemical properties (Kaur, Jain et al. 2009). This comes as no surprise due to the fact that nature has consistently shown to have much more complex and diverse molecules than any which can be achieved via synthetic approaches alone (Thomas and Johannes 2011). However, all of these drawbacks have a simple remedy made possible by synthetic strategies of modern day medicinal chemistry. During a process known as lead optimization, medicinal chemists are able to modify the original extract/alkaloid's primary chemical scaffold with various functional groups and/or chemical

(potentially nature-like) moieties. If successful, these modifications can potentially serve to improve the given compounds pharmacokinetic properties as well as antimalarial potency.

The seemingly endless amount of novelty displayed by nature should never be underestimated, and plants and animals alike should continue to be looked at for inspiration for nature-inspired synthetic libraries. Regardless of whether they may hold the cure for malaria or some other deadly disease, it is certain that so long as there is limited research and industrial disinterest within the field there will also be limited success.

Summary and Significance of Project

Despite the various efforts for malaria eradication, none have proven to be as effective as originally thought, and the discovery, characterization and development of novel inexpensive antimalarial therapeutics remains the world's best hope in one day eradicating the malaria parasite. Although numerous effective drugs have been developed thus far, resistance emergence has been documented to every single one, and this fact simply underscores the dire need for new compounds. The non-canonical life cycle of *Plasmodium* provides drug discovery researchers with a seemingly endless supply of potential targets. Likewise, the unprecedented chemical novelty provided by natural alkaloids and their semisynthetic nature-inspired derivatives provides the largest reservoir of compounds to potentially exploit and inhibit a specific part of the parasite's extensive life cycle. In accordance with these facts, this particular study aims to characterize potential lead compounds for their further development into next generation antimalarials.

CHAPTER TWO: MATERIALS AND METHODS

Asinex Nature-Inspired Chemical Libraries

In 2011, our drug discovery lab was given the unique oppurtunity to probe ASINEX's extremely diverse chemical libraries for compounds displaying high probability of antimalarial activity. The compound libraries, totaling over 70,000, were filtered based upon the following specifications: high oxygen content (preferably greater or equal to three oxygens), enriched with natural product-like chemical moieties, and absence of peroxide bridge(s). These specifications were selected for based upon the chemical qualities that have been displayed in other potent antimalarial compounds. This screen yielded 592 total compounds. However, the relatively recent screening of the Tres Cantos antimalarial compound set by GlaxoSmithKline led to the added specification of compounds which have high hydrophobicity indices (ClogP equal to or greater than 3.5) (Gamo, Sanz et al. 2010). Upon application of the additional hydrophobicity index preference, only 191 of the 592 compounds displayed the desired characteristics. These were ordered and screened against *P. falciparum* 3D7 (drug-sensitive) and Dd2 (multidrug resistant) strains. This preliminary screen identified six novel scaffolds with parasite potency within nanomolar range. More than six compounds displayed nanomolar potency, but similar scaffolds led to the selection of those that were the most potent. Considering the results from this preliminary parasite screening, the remaining 401 compounds were ordered and screened in the same manner. Upon their initial screening, three more novel scaffolds were discovered for a

grand total of nine unique structures. These nine compounds were designated as the parent compounds.

Culturing and Subculturing of NIH 3T3 Embryonic Mouse Fibroblasts

In order to determine the cytotoxic properties of these potential antimalarial compounds on mammalian cells, an embryonic mouse (*Mus musculus*) fibroblast cell line, more specifically NIH 3T3, was employed. This specific cell line was chosen for the cytotoxicity assays primarily because *Mus musculus* is the most commonly used animal research model, so preliminary cytotoxicity assay data with this cell line is expected to most adequately reflect cytotoxicity values which will be obtained in future mammal studies for these same compounds.

Culture guidelines set forth by the American Type Culture Collection (ATCC) were strictly followed. The cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) which was further modified to possess 4500 mg/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate and 10% v/v heat-inactivated newborn calf (bovine) serum. Furthermore, the cells were incubated and maintained in a humidified 5% carbon dioxide (CO_2) environment at 37ºC.

The cells were never allowed to achieve 100% confluency; in fact, the cells were strictly maintained below approximately 80% confluency. Once the culture achieved a near 80% confluency, the cells were then subcultured. Subculturing of NIH 3T3 fibroblasts involves

multiple steps. First, the old/used medium was removed using a Pasteur pipette. Next, the cells were washed with approximately 4 mL phosphate buffered saline (PBS) in order to remove any excess medium that contains proteins that act as a trypsin inhibitor in the next step if not removed. Once the cells have been adequately washed with PBS, they were treated with 1X trypsin-EDTA (~4 mL) for 30 seconds. After the initial 30 seconds, most of the trypsin was removed using a Pasteur pipette and the plate containing the cells was placed into a 37ºC incubator for 2 minutes to facilitate increased trypsin activity. The plate containing the cells was then removed from the incubator, and the cells were removed from the plate by resuspending them in approximately 7 mL of the modified DMEM described previously. A small portion of this culture resuspension, typically 1 mL, was retained in order to maintain the culture.

Promega CellTiter AQeous Non-Radioactive Cell Proliferation Assay

This primary cell proliferation/viability assay, also commonly called the MTS assay, utilizes a specific tetrazolium salt known as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) along with an electron-coupling reagent known as phenazine methosulfate (PMS) in order to measure dehydrogenase activity which is only observed in metabolically active (viable) cells. In viable/living cells, dehydrogenase activity causes the reduction of MTS into a purple formazan crystal product that is soluble in cell tissue culture media. Subsequently, the presence of this formazan crystal product can be assayed by measuring absorbance at 492 nm. Considering that the amount of

formazan present is directly proportional to the quantity of viable cells, the absorbance values measured and dose-response curve generated can be used to extrapolate the half maximal inhibitory concentration or cytotoxic effect of specific compounds on the given mammalian cell line.

For this particular cell viability assay, NIH 3T3 embryonic mouse fibroblast cells were added to a 384-well tissue treated transparent bottom plate at an approximate cellular concentration of 2,500 cells per well for a total volume of 25 μL per well. These cell seeded plates were then placed into a 37ºC, 5% CO_2 for 24 hours in order to facilitate attachment of the fibroblasts as well as limited proliferation. After this initial 24 hour period, the plates were removed and the compounds which were to be assayed were added to each well in triplicate manner. Three separate concentrations were used for each given compound: 1 μM, 10 μM and 100 μM in order to generate a dose-response curve from which half maximal inhibitory concentration can be extrapolated. The positive control that was used for this assay was 60 ng/μL digitonin. Additionally, DMSO controls of 1% v/v, 0.75% v/v and 0.5% v/v were used to negate any toxicity by the compounds' solvent. Once the appropriate amount of compound had been added to each well, the plate was then placed back into the incubator where it was left for 48 hours. After this 48-hour incubation time, the plate was removed from the incubator and approximately 10 μL of MTS solution/reagent was added to each well. The plate was then placed back into the incubator for approximately 2 hours to allow the assays reaction to take

place. Immediately following this final incubation period, the plate was again removed from the incubator and placed in a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader in order to measure absorbance at 492 nm. The absorbance results were then extracted into an appropriately labeled Microsoft Excel spreadsheet and analyzed using GraphPad's Prism software.

Promega Kinase-Glo® Luminescent Kinase Assay

In order to confirm the validity of the previously described MTS assay in antimalarial cytotoxicity screening, Promega's Kinase-Glo® assay has been employed. Fundamentally, this assay takes advantage of a reaction that is catalyzed by the enzyme known as luciferase, specifically Ultra-Glo™ Recombinant Luciferase. Although this assay is not specifically intended for assaying cytotoxicity, the luciferase enzyme being used can be combined with a specific 'luciferase-safe' cell culture lysis reagent (CCLR) from the manufacturers separate Luciferase Assay System to lyse a cell culture while not interfering with the luciferase reaction. When a cell culture is used in combination with Kinase-Glo® luciferase and substrate as well as a specific buffer and cell culture lysis reagent (all of which have been deemed proprietary), the result is a reaction between the ATP from the cell culture lysate and the Kinase-Glo® solutions that results in the production of a measurable luminescent signal. This reaction occurs only in the presence of ATP that is found solely in metabolically active (or viable) cells, and, thus, it can be used to assay the selectivity of potential antimalarial compounds. Additionally, the specific

luciferase reaction provided by this system has been proven to be compatible (by the manufacturer) with the ASINEX compounds primary solvent, DMSO (dimethylsulfoxide).

This modified Kinase-Glo® assay was achieved in combination with the same culture of NIH 3T3 embryonic mouse fibroblasts used in the previously described MTS assay. Initially, the fibroblast culture was diluted to an approximate concentration of 50,000 cells/mL; the cellular concentration was determined using trypan blue exclusion with a hemacytometer. This suspended culture was then aliquoted into a 384-well solid white tissue culture-treated plate in such a way that each well contained a total volume of 50 μL, or approximately 2,500 cells per well. These plates were then incubated in a humidified 37°C, 5% CO_2 environment for 24 hours to facilitate fibroblast attachment. Next, the compounds to be assayed were added in triplicates of the following concentrations: 100 μM, 75 μM, 50 μM, 25 μM and 10 μM. Negative controls were employed in the form of wells containing only the modified DMEM cell culture medium (no cells) and wells containing suspended culture in the absence of any compound or inhibitor. The positive control wells contained the known cytotoxic agent digitonin (20 mg/mL). Additionally, DMSO controls were used at concentrations of 1% v/v, 0.75% v/v, and 0.5% v/v to account for any cytotoxicity conveyed by the compounds primary solvent. After all compounds and controls were properly added, the plates were returned to the incubator for an additional 48 hours. After this incubation period, each well's volume was aspirated so that only the attached (viable) fibroblasts remained and the wells were washed with a PBS solution. Next, the cells

were treated with 20μL of the CCLR and shaken for 15 minutes to ensure adequate cell lysis. The Kinase-Glo® reagents were than added in 1:1 ratio (20 μL) to the cell lysate for a final well volume of 40 μL. The plates were shaken for an additional 10 minutes which was followed by measuring luminescence with a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader. The results were subsequently analyzed with Microsoft Excel and GraphPad Prism software.

Generation of Drug Resistant Parasite Lineage and Parasite Culturing

The generation of a drug resistant parasite line is an approach used for the determination of drug-specific parasite resistance potential and drug target identification. In order to generate this resistant parasite, a known multidrug resistant strain of *Plasmodium falciparum*, Dd2, was employed. The strain was subjected to the most potent antimalarial compound discovered within the Asinex nature-inspired chemical libraries via two separate approaches deemed the step-wise approach and the intermittent approach. In order to increase the likelihood of resistance development and facilitate the parasite's mutation rate, the parasite was grown up to a point where there was a total number of infected erythrocytes equaled approximately 10^{10} prior to the first administration of compound. The Dd2 parasite strain was originally derived from the W2 strain, and the W2 strain has been shown to develop drug resistance to other inhibitors when as little as 10^6 infected erythrocytes are initially used (Certain and Sibley 2007; Gassis and Rathod 1996).

For the step-wise approach, the asynchronous parasite population was continuously subjected to a concentration of drug that gradually increased as time progressed. First, the population was treated with the predetermined IC_{25} of the given compound each day for 10 days.

After this initial 10 day treatment, the dosage was increased to the predetermined IC_{90}. This dosage was administered each day for approximately 2 weeks or until a parasitemia (percent of infected erythrocytes) of 1-2% was achieved. The concentration was then increased to 5 x IC_{90} until a healthy, resistant parasite lineage emerged. Additionally, the given cultures were treated with the specific compound with each medium change.

For the intermittent approach, the asynchronous parasite population was treated with 5 x IC_{90} in an intermittent manner. The initial population was treated with the specified amount for seven days in order to ensure at least three generations of parasite were subjected to the compound during each round of treatment. After a week of treatment at this very high concentration, the parasitemia was, as expected, reduced to a value which was nearly unidentifiable. The treatment was then stopped to allow the population to return to a parasitemia of 2-4%. Once this occurred, another round of treatment was administered, and the process repeated itself until a healthy, resistant parasite lineage emerged. Additionally, the cultures were treated with the specific compound with each medium change during each seven day cycle.

The Dd2 culture was maintained in a modified RPMI 1640 medium, collectively known as RP0.5A, with the following composition: 1X RPMI 1640, 0.5% w/v Albumax, 300 mg/L L-glutamine, 25 mM HEPES, 0.2% w/v dextrose, 0.2% w/v sodium bicarbonate, 15 mg/L hypoxanthine and 25 μg/mL gentamycin. In addition, the culture was maintained at a 4% hematocrit with washed type A+ blood from the local Florida Blood Center. The medium was changed everyday to ensure healthy parasites.

Invitrogen SYBR Green I-Based Fluorescence Assay

This fluorescence-based assay was used to determine and compare the half maximal inhibitory concentrations of the most effective compound discovered in the Asinex libraries on the DD2 *P. falciparum* strain before and after the drug resistance development approaches, and on Dd2 and 3D7 to determine relative IC_{50}s of the parent compounds and their respective analogs. It utilizes a special cyanine dye called SYBR Green-I that acts as a DNA intercalating agent. Fundamentally, the assay exploits the fact that erythrocytes lack nucleic acids, whereas *Plasmodium* does not. Since the culture being assayed contained only *Plasmodium* parasites and erythrocytes, the only DNA present belonged solely to the parasite. After complex formation between the dye and DNA, a fluorescent signal is generated which can be measured at 520 nm using a microplate reader. The fluorescence measurements obtained have been shown to be directly proportional to the level of parasitemia over a wide range of parasitemias. With this data graphed upon a dose-response curve, the elucidation of a specific compound's IC_{50} is made possible. Validation for this assay has been extensively considered, and past studies have proven time and time again that is more than adequate for high throughput screening antimalarial assays (Bennet, Paguio et al. 2004; Johnson, Dennull et al. 2007; Co, Dennull et al. 2009).

This assay allows the use of only one reagent that contains both the SYBR Green-I dye as well as cell lysis components. Initially, the lysis buffer was prepared to the following specifications: 20 mM Tris pH 7.5, 5 mM EDTA, 0.008% w/v saponin and 0.08% v/v Triton X-

100. Immediately before the buffer was added to the plates to initiate the assay, 1 μL of 10000X SYBR Green-I dye was added per 10 mL of lysis buffer for a 1X final concentration of dye. Due to the light sensitive nature of SYBR Green-I, precautions were taken to limit the dye's exposure to direct light.

In order to prepare the parasites for this assay, the original culture was first Giemsa stained and examined microscopically to determine the approximate level of parasitemia. In addition, a small volume of culture was also loaded into a hemacytometer in order to microscopically determine the culture's hematocrit. Once accurate values for both parasitemia and hematocrit were obtained, calculations were made so that the culture's parasitemia could be reduced to 1% and the hematocrit diluted to 2%. Immediately after preparing the culture, the drug dilution plates were prepared by aliquoting (in triplicates) the required amount of compound into their respective wells. The final concentrations included 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM for the resistant parasite line assay plate and 0.1 μM. 0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM for all preliminary assays of the Asinex compounds. The control used for each SYBR assay was 10 μM chloroquine. The dilution plates were constrained to only four concentrations for the drug resistant line due to limitations in compound (UCF201) availability. The previously modified culture was then pipetted into a 96-well black bottom plate so that each well contained a final culture volume of 100 μL. The plate was then allowed to incubate in a humidified 37°C, 5% CO_2 environment for 72 hours to ensure compound interaction with the parasite's complete erythrocytic life cycle (~48 hours). Following this incubaiton period, the plates were placed in a -80°C freezer overnight. The following day the plates were thawed and subsequently lysed with

the aforementioned SYBR lysis buffer. To adequately lyse all red blood cells, 100 μL of buffer was added to each well, and the plate was incubated for 30 minutes. Immediately following lysis, the plates were measured for fluorescence at 520 nm with a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader. Data from the plate reader was then extracted into a Microsoft Excel spreadsheet and GraphPad Prism for analysis.

CHAPTER THREE: RESULTS

Selectivity Determination of Various Nature-inspired Synthetic Compounds

One of the most challenging obstacles presented to drug discovery researchers is the discovery of a drug that not only displays potent activity against the organism of interest but one that also displays a high selectivity for that organism. In light of this fact, the primary objective of this project was to assay the selectivity indices of the compounds obtained from the ASINEX chemical libraries.

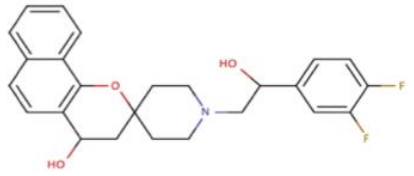
Validation of these assays was performed in numerous manners. The first of which was an overall accuracy validation via standard score (also commonly referred to as Z-factor) determination. The Z-factor is obtained via the following equation: $Z = 1 - 3((\sigma_{c+} + \sigma_{c-}) / |\eta_{c+} - \eta_{c-}|)$ where σ_{c+} is standard deviation of the positive controls, σ_{c-} is standard deviation of negative controls, η_{c+} is the average value of the positive controls and η_{c-} is the average value of the negative controls (Zhang, Chung et al. 1999). This value is universally used in most research involving high throughput screening assays, and it ultimately determines the validity and accuracy of the particular assay. The Z-factor was determined to be an average of 0.9465 for all MTS assays performed. According to current standards in high throughput screenings, a Z-factor equal to or greater than 0.5 represents an assay that is accurate enough for use in screening hits (Zhang, Chung et al. 1999). The second confirmation of assay validity was provided via the modified Kinase-Glo assay described in Chapter Two. Upon n=3 determination, the average Z-factors proved to be slightly less than with the MTS assay and equal to 0.8544. However, this value was still over the recommended 0.5 thus validating it's use. The results of the modified Kinase-Glo assay on the ASINEX parent compounds corresponded with the results given by the

MTS assays for the same compounds. Correspondence of the two results ultimately confirms the validity of the MTS assay as the primary cytotoxicity assay.

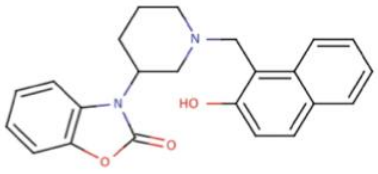
The parent compounds were selected based upon their demonstration of >70% parasite inhibition at 10 μM. From this initial parameter, nine of the most potent chemical scaffolds were discovered. Following their discovery, their chemical structures were analyzed in comparison to the other 583 compounds within the obtained libraries, leading to the subsequent discovery of 104 total analogs. The exact number of analogs per parent compound is represented in Table 1 below. Additionally, the chemical structures along with their IUPAC nomenclature for each parent compound are shown in Figure 1.

Parent Compound (UCF I.D. / ASINEX I.D.)	Number of Analogs
UCF 201 / LEG17217214	24
UCF 301 / AEM12673344	3
UCF 401 / AEM18334709	7
UCF 501 / BAS00327426	1
UCF 601 / BDC24040139	8
UCF 701 / SYN23011295	4
UCF 801 / BDD24084121	35
UCF 901 / BAS01260479	3
UCF 1001 / SYN15412666	19
Total Number of Analogs	104

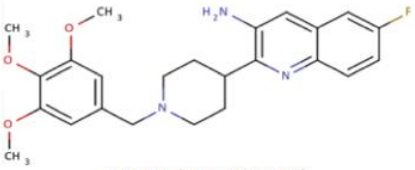
Table 1. Each parent compound along with their respective number of structural analogs.



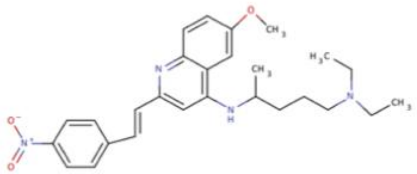
UCF 201 (LEG17217214)
1'-[2-(3,4-difluorophenyl)-2-hydroxyethyl]spiro[3,4-dihydrobenzo[h]chromene-2,4'-piperidine]-4-ol



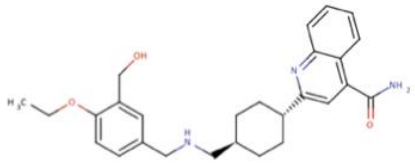
UCF 301 (AEM12673309)
3-{1-[(2-hydroxynaphthalen-1-yl)methyl]piperidin-3-yl}-2,3-dihydro-1,3-benzoxazol-2-one



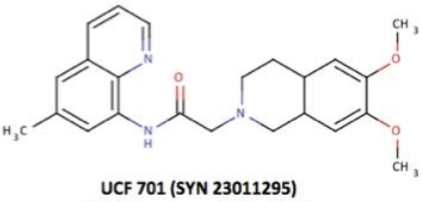
UCF 401 (AEM 18334709)
6-fluoro-2-{1-[(3,4,5-trimethoxyphenyl)methyl]piperidin-4-yl}quinolin-3-amine



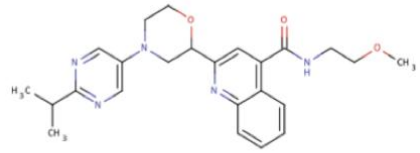
UCF 501 (BAS 00327426)
diethyl[4-({6-methoxy-2-[(E)-2-(4-nitrophenyl)ethenyl]quinolin-4-yl}amino)pentyl]amine



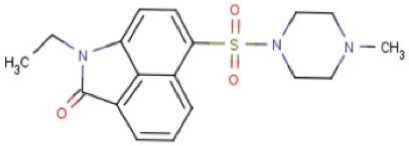
UCF 601 (BDC 24040139)
2-[(1r,4r)-4-[({[4-ethoxy-3-(hydroxymethyl)phenyl]methyl}amino)methyl]cyclohexyl]quinoline-4-carboxamide



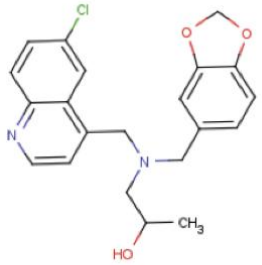
UCF 701 (SYN 23011295)
2-(6,7-dimethoxy-1,2,3,4,4a,8a-hexahydroisoquinolin-2-yl)-N-(6-methylquinolin-8-yl)acetamide



UCF 801 (BDD 24084121)
N-(2-methoxyethyl)-2-{4-[2-(propan-2-yl)pyrimidin-5-yl]morpholin-2-yl}quinoline-4-carboxamide



UCF 901 (BAS 01260479)
2-ethyl-9-(4-methylpiperazine-1-sulfonyl)-2-azatricyclo[6.3.1.0^{4,12}]dodeca- 1(11), 4(12),5,7,9-pentaen-3-one



UCF 1001 (SYN 15412666)
1-[(2H-1,3-benzodioxol-5-ylmethyl)[(6-chloroquinolin-4-yl)methyl]amino]propan-2-ol

Figure 1. Chemical structures and IUPAC nomenclature of each parent compound.

The first compounds to be screened for cytotoxicity against 3T3 cells were the parent compounds. Each compound was assayed in triplicate concentrations on three separate occasions (n=3) with the average Z-factor being equal to 0.9465. The average parasite (Dd2) half maximal inhibitory concentration (IC_{50}) and mammalian (NIH 3T3) half maximal effective concentrations (EC_{50}) are shown in Table 2 along with their respective selectivity indices (SI). However, the selectivity indices were not able to be accurately determined for UCF401, UCF701 and UCF801 because the mammalian cell EC_{50}s for these compounds were never reached. Additionally, the parent compounds which exhibited a mammalian cell EC_{50} of <100 μM have their responses plotted in Figure 2.

ASINEX I.D.	UCF I.D.	Dd2 EC_{50} (μM)	NIH 3T3 EC_{50} (μM)	SELECTIVITY INDEX	*VIABLE CELLS AT 100 μM (%)
LEG 17217214	UCF 201	0.39	47	121	N/A
AEM 12673344	UCF 301	0.39	63	161	N/A
AEM 18334709	UCF 401	0.45	>100	>222*	73
BAS 00327426	UCF 501	0.48	22	46	N/A
BDC 24040139	UCF 601	0.61	38	62	N/A
SYN 23011295	UCF 701	0.72	>100	>138*	85
BDD 24084121	UCF 801	0.40	>100	>250*	90
BAS 01260479	UCF 901	0.44	68	154	N/A
SYN 15412666	UCF 1001	0.66	76	115	N/A

Table 2. Parasite (Dd2) IC_{50}s and mammalian cell (NIH 3T3) EC_{50}s along with their corresponding selectivity indices. Parasite IC_{50}s were determined via the SYBR Green-I assay with a 10 μM CQ control. Mammalian cell EC_{50}s were determined via the MTS assay with a 60 ng/μL digitonin control. Selectivity indices are the ratio of NIH 3T3 EC_{50} to DD2 IC_{50}. * indicates that the selectivity index for the given compound could not be accurately determined due to the fact that the mammalian cell EC_{50} was never achieved. However, the percentage of viable cells that were present at 100 μM is displayed in the last column of the table.

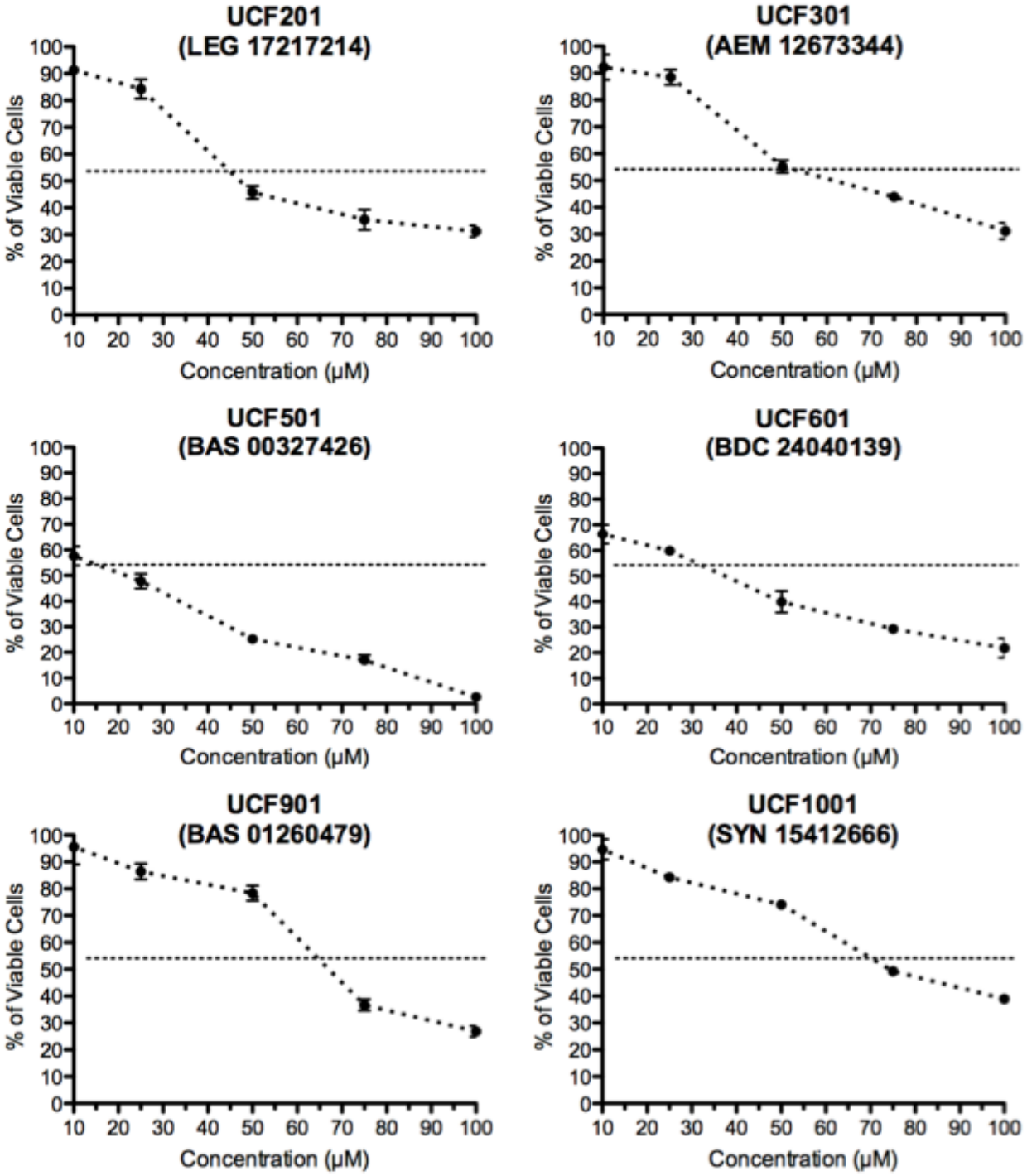


Figure 2. NIH 3T3 fibroblast toxicity in the presence of UCF201, UCF301, UCF501, UCF601, UCF901 and UCF1001. Cytotoxicity was determined using the following concentrations: 10 μM, 25 μM, 50 μM, 75 μM and 100 μM. 100 μM was the maximum concentration utilized due to toxic DMSO concentrations above this point.

Subsequent screening involved the analogs of each respective parent compound. The average parasite IC_{50} and mammalian EC_{50} results along with the corresponding selectivity indices of each analog in each series are represented in Table 3. The average Z-factor for all of the assays performed was 0.9465, an excellent score for high throughput screening experiments.

ASINEX I.D.	UCF I.D.	Dd2 EC_{50} (μM)	NIH 3T3 EC_{50} (μM)	SELECTIVITY INDEX	*VIABLE CELLS AT 100 μM (%)
LEG 17216981	UCF 202	0.47	51	109	N/A
LEG 17216993	UCF 203	0.99	50	51	N/A
LEG 17217077	UCF 204	1.3	54	42	N/A
LEG 17217088	UCF 205	0.53	53	100	N/A
LEG 17217110	UCF 206	0.59	64	110	N/A
LEG 17217203	UCF 207	0.75	53	71	N/A
LEG 17217272	UCF 208	0.68	50	74	N/A
LEG 17217283	UCF 209	0.48	51	106	N/A
LEG 17217352	UCF 210	0.47	52	111	N/A
LEG 17217066	UCF 211	0.93	53	57	N/A
LEG 17217140	UCF 212	0.78	53	68	N/A
LEG 17217152	UCF 213	0.59	53	90	N/A
LEG 17217173	UCF 214	0.90	52	58	N/A
LEG 17217174	UCF 215	1.1	52	46	N/A
LEG 17217175	UCF 216	1.0	51	50	N/A
LEG 17217176	UCF 217	0.66	52	80	N/A
LEG 17217177	UCF 218	1.3	54	42	N/A
LEG 17217178	UCF 219	0.82	50	61	N/A
LEG 17217179	UCF 220	0.83	51	61	N/A
LEG 17217180	UCF 221	1.2	53	45	N/A
LEG 17217230	UCF 222	0.94	54	58	N/A
LEG 17217309	UCF 223	0.64	52	81	N/A
LEG 17217321	UCF 224	0.79	51	65	N/A
LEG 17217341	UCF 225	0.72	51	71	N/A
AEM 12673344	UCF 302	0.62	63	100	N/A
AEM 12674533	UCF 303	0.57	63	111	N/A
BAS 04246204	UCF 304	0.51	>100	>196*	90
BDC 29760189	UCF 402	0.88	>100	>114*	85

LMK 17223586	UCF 403	0.94	>100	>106*	86
LMK 17223587	UCF 404	1.1	>100	>91*	78
LMK 17223596	UCF 405	1.1	>100	>91*	80
LMK 17223597	UCF 406	0.73	>100	>137*	85
LMK 17224176	UCF 407	0.85	>100	>118*	84
LMK 17224177	UCF 408	0.71	>100	>141*	91
BAS 00897438	UCF 502	1.1	>100	>91*	86
BDC 24040096	UCF 602	0.69	66	96	N/A
BDC 24040199	UCF 603	1.0	>100	>96*	83
LMK 17223586	UCF 604	0.73	>100	>137*	91
LMK 17223587	UCF 605	1.2	>100	>86*	87
LMK 17223596	UCF 606	1.3	>100	>80*	72
LMK 17223597	UCF 607	0.63	>100	>158*	92
LMK 17224176	UCF 608	0.90	>100	>111*	95
LMK 17224177	UCF 609	0.69	>100	>146*	89
SYN 22931911	UCF 702	1.0	>100	>100*	80
SYN 22981169	UCF 703	1.1	>100	>89*	85
SYN 22981198	UCF 704	1.1	>100	>91*	77
BAS 00371682	UCF 705	0.83	>100	>120*	81
BDD 26909562	UCF 802	0.54	>100	>187*	93
BDD 27574625	UCF 803	0.90	>100	>111*	96
BDD 24084208	UCF 804	0.63	>100	>159*	79
BDD 24084210	UCF 805	0.48	>100	>210*	68
BDD 24084211	UCF 806	0.64	>100	>156*	72
BDD 24084265	UCF 807	0.48	>100	>207*	96
BDD 24084289	UCF 808	0.31	>100	>326*	92
BDD 24084590	UCF 809	0.88	>100	>114*	77
BDD 24084591	UCF 810	0.79	>100	>125*	82
BDD 24084610	UCF 811	0.94	>100	>107*	79
BDD 24084927	UCF 812	0.74	>100	>135*	76
BDD 24084928	UCF 813	0.43	>100	>233*	98
BDD 24085323	UCF 814	0.34	>100	>294*	76
BDD 24085406	UCF 815	0.46	>100	>217*	85
BDD 24085437	UCF 816	0.44	>100	>227*	69
BDD 24085693	UCF 817	0.54	>100	>184*	77
BDD 24085863	UCF 818	0.64	>100	>157*	80
BDD 24085693	UCF 819	0.98	>100	>102*	95
BDD 24085895	UCF 820	0.68	>100	>146*	87

BDD 24085896	UCF 821	0.67	>100	>148*	83
BDD 24085897	UCF 822	0.44	>100	>227*	56
BDD 26908750	UCF 823	0.74	>100	>135*	81
BDD 26908751	UCF 824	0.94	>100	>106*	92
BDD 26909379	UCF 825	0.49	>100	>204*	99
BDD 26909472	UCF 826	0.54	>100	>185*	77
BDD 26909560	UCF 827	0.53	>100	>190*	78
BDD 26909561	UCF 828	0.65	>100	>154*	77
BDD 24084414	UCF 829	0.41	>100	>244*	97
BDD 24084415	UCF 830	0.47	>100	>212*	89
BDD 24084417	UCF 831	0.64	>100	>155*	79
BDD 24083352	UCF 832	0.59	>100	>168*	85
BDD 24084049	UCF 833	0.52	>100	>191*	96
BDD 24084107	UCF 834	0.58	>100	>172*	96
BDD 24084048	UCF 835	0.83	>100	>120*	97
BDD 24084119	UCF 836	0.63	>100	>160*	74
BAS 01126330	UCF 902	1.3	>100	>77*	86
BAS 00460241	UCF 903	0.66	>100	>151*	87
BAS 00460273	UCF 904	0.78	>100	>128*	81
SYN 15412684	UCF 1002	0.73	>100	>137*	96
SYN 15412656	UCF 1003	0.69	>100	>144*	99
SYN 15412520	UCF 1004	0.99	>100	>101*	95
SYN 15412490	UCF 1005	0.82	>100	>121*	90
SYN 15412489	UCF 1006	0.84	>100	>119*	91
SYN 15412386	UCF 1007	0.78	>100	>128*	84
SYN 15412385	UCF 1008	0.71	>100	>140*	94
LMG 09142618	UCF 1009	1.1	>100	>88*	59
LMG 09142611	UCF 1010	1.1	>100	>93*	60
LEG 15411875	UCF 1011	0.71	>100	>140*	93
LEG 15411860	UCF 1012	0.85	>100	>117*	86
BAS 12416291	UCF 1013	0.69	>100	>145*	84
BAS 01313367	UCF 1014	1.0	>100	>100*	91
BAS 01124611	UCF 1015	0.83	>100	>120*	99
BAS 01124862	UCF 1016	0.91	>100	>109*	95
BAS 01124990	UCF 1017	0.82	>100	>121*	90
BAS 01124993	UCF 1018	0.86	>100	>115*	91
BAS 01125425	UCF 1019	0.91	>100	>109*	92
BAS 01125538	UCF 1020	0.99	>100	>101*	89

Table 3. Parasite (Dd2) and mammalian cell (NIH 3T3) EC_{50}s along with their corresponding selectivity indices for all parent compound analogs. Parasite IC_{50}s were determined via the SYBR Green-I assay with a 10 μM CQ control. Mammalian cell EC_{50}s were determined via the MTS assay with a 60 ng/μL digitonin control. Selectivity indices are the ratio of NIH 3T3 EC_{50} to DD2 IC_{50}. * indicates that the selectivity index for the given compound could not be accurately determined due to the fact that the mammalian cell EC_{50} was never achieved. However, the percentage of viable cells that were present at 100 μM is displayed in the last column of the table.

Potential for Novel Drug Resistant Parasite Lineage

The most potent compound tested in the ASINEX libraries obtained, UCF201 (LEG17217214), was also used to assay it's potential for drug resistance development against the multi-drug resistant strain of *P. falciparum* Dd2. Initially, the IC_{50} was determined to be 391 nM, shown in Figure 3. For comparison, the original IC_{50} was also determined in a drug sensitive strain of *P. falciparum* known as 3D7. The IC_{50} in 3D7 was proven to be 402 nM, a value that is sufficiently similar to that in Dd2 to prove no significant difference exists between resistant and sensitive phenotypes.

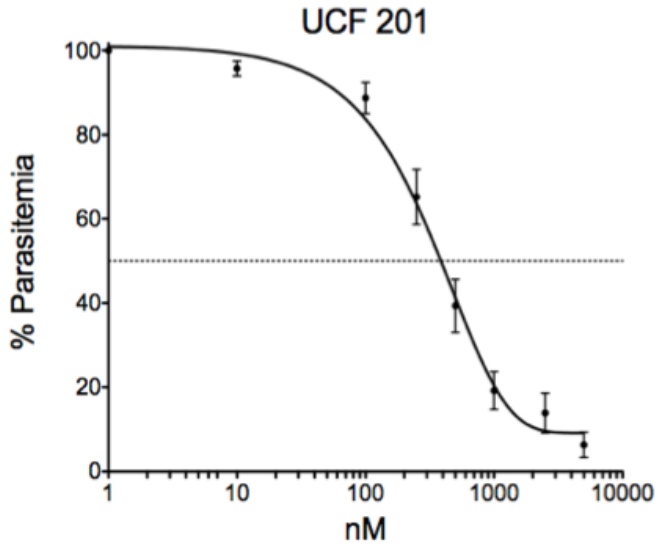


Figure 3. Initial inhibition of *P. falciparum* Dd2 parasite growth by UCF201 (LEG17217214) via the SYBR Green-I assay. The initial screening concentrations were 10 nM, 100 nM, 250 nM, 500 nM, 1000 nM, 2500 nM and 5000 nM with a 10 μM CQ control. The intersection between the 50% parasitemia grid line and the dose-response curve is UCF201's IC_{50} (391 nM).

Subsequent screening of the parasite that was treated with compound via the intermittent approach was performed during day 38 (following three seven-day cycles of treatment). This secondary screening demonstrated an IC_{50} equal to 4.411 μM, shown in Figure 4. This corresponded to an 11.28-fold increase in the parasite's original IC_{50}. The intermittent resistant

Dd2 was assayed instead of the step-wise resistant strain for two particular reasons. First, during treatment of both cultures it became obvious that the intermittent approach was seeing much more success than the step-wise approach. This conclusion was reached based upon the 5X IC_{90} results being displayed. Upon treatment of both cultures with the same concentration of compound, the step-wise approach was seeing much more inhibition than the intermittent approach. This was suggestive that a greater resistance has been generated in the intermittent approach. Additionally, the availability of UCF201 at the time of screening limited application of the assay to only one resistant line approach.

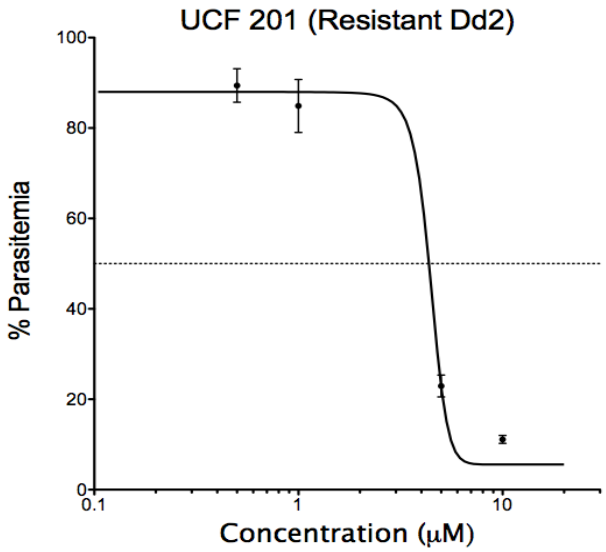


Figure 4. Inhibition of resistant *P. falciparum* Dd2 parasite growth by UCF201 (LEG17217214) via the SYBR Green-I assay. The screening concentrations used were 0.5 μM, 1 μM, 5 μM and 10 μM with a 10 μM CQ control. The intersection between the 50% parasitemia grid line and the dose-response curve is UCF201's IC_{50} (4.411 μM). Only four concentrations (as opposed to seven in the initial screen) were used due to a limited quantity of the UCF201 compound at the time of screening.

The resistant parasite line was also screened against the known antimalarials CQ, MQ, ART and PYR to determine if there were any fluctuations in the compounds IC_{50}s against Dd2. The results are presented in Table 4.

Standard Inhibitor	Original Dd2 IC_{50} (nM)	Resistant Dd2 IC_{50} (nM)	Fold Increase
Chloroquine (CQ)	382±44	402±67	0.05
Mefloquine (MQ)	53±17	67±8	0.26
Artemisinin (ART)	11±7	14±2	0.27
Pyrimethamine (PYR)	2519±113	2718±214	0.08

Table 4. Comparison of standard inhibitor IC_{50}s against the resistant Dd2 parasite. The original Dd2 IC_{50}s were determined in the same Dd2 strain which was used to generate the resistant Dd2 phenotype. The resistant Dd2 IC_{50}s were determined via the SYBR Green-I assay.

CHAPTER FOUR: DISCUSSION

Compound Selectivity and Significance of Work

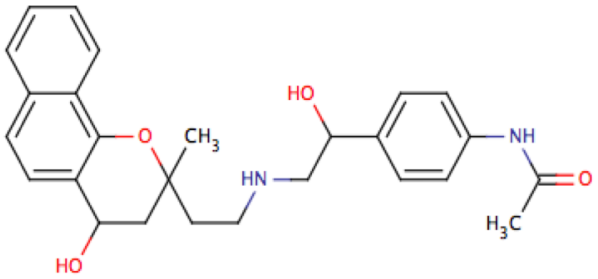
In all drug discovery research, selectivity index determination is key in preliminary hit screenings. Fundamentally, the selectivity index of a compound is a measure of its ability to specifically inhibit a certain organism, *P. falciparum* in this study, while leaving the host of the organism unharmed or minimally affected. More specifically, the selectivity index of a given compound is defined as the ratio between the *in vitro* mammalian cell (NIH 3T3) EC_{50} and the *in vitro* parasite (*P. falciparum* Dd2) IC_{50}. In accordance with the importance of selectivity determination, the relative selectivity indices of the most potent ASINEX compounds and their structural analogs was determined and reported herein.

According to the MMV's criteria for hit compound progression, an acceptable hit compound IC_{50} for cellular parasite (Dd2) assays is less than 1 μM. Additionally, the same guidelines indicate that an acceptable hit compound selectivity index is greater than ten. By following the above parameters, it is possible to evaluate the data presented in Tables 2 & 3 and to determine which ASINEX compounds are most promising for use in future studies.

Despite each possessing a SI greater than ten, compounds 204, 215, 216, 218, 221, 404, 405, 502, 603, 605, 606, 703, 704, 902, 1009, 1010, and 1014 can be eliminated from future studies based upon their demonstration of parasite IC_{50}s greater than 1 μM.

All other compounds that were screened met the guidelines set forth by the MMV's criteria for drug progression; each compound displayed a parasite IC_{50} less than 1 μM and a SI of greater than ten.

Analysis of the UCF201 series displayed the most overall cytotoxic compounds in the library. The minimum EC_{50} (most cytotoxic or least selective) displayed within this series was by the parent compound itself (UCF201) at 47 μM, whereas the maximum EC_{50} (least cytotoxic or most selective) was 64 μM displayed by UCF206. In an attempt to elucidate possible explanations for this slight difference, their chemical structures were empirically analyzed. The difference could be due to the absence of the piperidine ring in UCF206 (Figure 5) as compared to it's presence within each of the other compounds; however, confirmation of this theory will need to be made in any future studies involving these specific compounds.



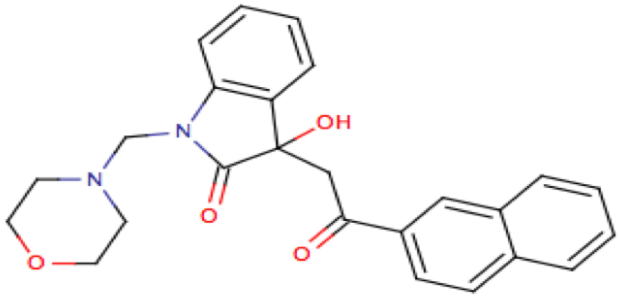
UCF 206 (LEG 17217110)

N-(4-{1-hydroxy-2-[(2-{4-hydroxy-2-methyl-2H,3H,4H-naphtho[1,2-b]pyran-2-yl}ethyl)amino]ethyl}phenyl)acetamide

Figure 5. Chemical structure and IUPAC nomenclature for UCF206.

The majority of the 301 series (3 out of 4) showed EC_{50}s in the low 60 μM range. However, the last compound of the series, UCF304, displayed an EC_{50} of >100 μM. Examination of each compounds chemical structures to determine probable causes for the observed difference showed that UCF304 (Figure 6) has a morpholine ring and lacks a piperidine

ring, whereas the other compounds in the family lack a morpholine ring but possess a piperidine ring. However, whether or not the presence or absence of the specified rings conveyed UCF304s marked difference in cytotoxicity remains to be determined.

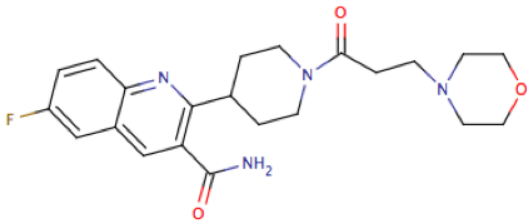


UCF 304 (BAS 04246204)

3-hydroxy-1-(morpholin-4-ylmethyl)-3-[2-(naphthalen-2-yl)
-2-oxoethyl]-2,3-dihydro-1H-indol-2-one

Figure 6. Chemical structure and IUPAC nomenclature for UCF304.

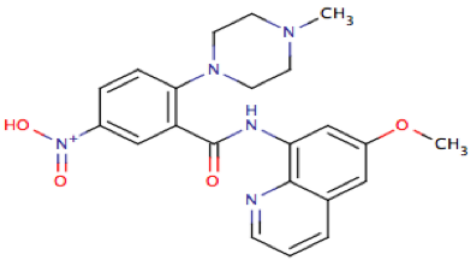
Analysis of the UCF401 analogs showed no significant variations among parasite IC_{50}s or mammalian cell EC_{50}s. However, there was a marked difference in the parent compound's (UCF401) parasite IC_{50} compared to that of the analogs. Each analog has a parasite IC_{50} that is slightly greater than that of the parent. Comparison of their chemical structures revealed that the parent compound is the only one in the family that lacks a morpholine ring (UCF408 shown in Figure 7 for comparison). However, whether the absence of this heterocyclic ring increased its antimalarial potency as opposed to other members of the family should be determined in future studies.



UCF408 (LMK17224177)
6-fluoro-2-{1-[3-(morpholin-4-yl)propanoyl]piperidin-4-yl}quinoline-3-carboxamide

Figure 7. Chemical structure and IUPAC nomenclature for UCF408.

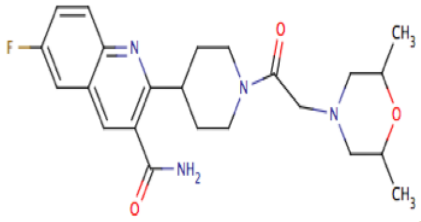
The 501 series of compounds contained the most cytotoxic compound out of the entire set of compounds screened. UCF501 displayed an average EC_{50} of 22 μM corresponding to a selectivity index of 46; however, the only other compound within the series (UCF502, Figure 8) showed a dramatic increase in both the parasite IC50 and the mammalian cell EC50, as shown in Table 3. Upon analysis of their structures, the only large structural difference between the two was the presence of a piperazine ring in UCF502 while it was lacking in UCF501. Despite this difference, it must still be confirmed in future studies whether the rings presence in UCF502 was the underlying cause of the variations seen.



UCF502 (BAS00897438)
5-(hydroxynitroso)-N-(6-methoxyquinolin-8-yl)-2-(4-methylpiperazin-1-yl)benzamide

Figure 8. Chemical structure and IUPAC nomenclature for UCF502.

The UCF601 series proved to be an overall selective batch of compounds barring only two structures. All compounds in this series except UCF601 and 602 demonstrated a consistent EC_{50} of greater than 100 μM; UCF601 and 602 respectively displayed EC_{50}s of 38 μM and 66 μM that corresponded to selectivity indices of 62 and 96, respectively. Furthermore, the only chemical difference that could be empirically determined was the absence of the heterocyclic morpholine ring in compounds 601 and 602 (UCF607 displayed in Figure 9 for comparison). However much like the other structural differences exhibited in the other series, the absence of the morpholine ring may not necessarily be responsible for the observed differences.



UCF607 (LMK17223597)
2-{1-[2-(2,6-dimethylmorpholin-4-yl)acetyl]piperidin-4-yl}-6-fluoroquinoline-3-carboxamide

Figure 9. Chemical structure and IUPAC nomenclature of UCF607.

The UCF701 and UCF801 families displayed no significant variation among their respective parents and analogs, and, in accordance with this fact, structural analysis of each was not performed.

Both the 901 and 1001 series showed excellent selectivity indices in all compounds. UCF901 and UCF1001 displayed EC_{50}s of 68 μM (SI of 154) and 76 μM (SI of 115), respectively; all others in both groups showed EC_{50}s of greater than 100 μM. Despite the differences seen between the parents and their respective analogs, no structural differences could be determined which suggests other non-empirical properties are most likely responsible.

However, validation of this theory must be performed in any future studies involving these specific compounds.

This study set the stage for the next step that must occur to validate each compound as a hit. Following the conclusions that were made, the compounds that met the hit screening guidelines for parasite IC_{50} and SI should be further analyzed via the elucidation of their specific ADME (Absorption Distribution Metabolism and Excretion) profiles. This pharmacokinetic profile is the final step in hit validation, and, upon adequate ADME demonstration, the most promising hits can be upgraded in status to an early lead compound. As a part of a separate study in our drug discovery lab, ADME profiles of parent compounds UCF201, UCF301 and UCF401 have already been determined. The UCF201 and UCF401 ADME profiles proved to be extremely promising, a fact that will ultimately lead to further studies involving these particular compounds. However, UCF301's ADME profile demonstrated its solubility as well as microsomal stability are inadequate for a lead compound, and it was thus eliminated from any future studies.

Probability of Drug Resistance and Possible Target Elucidation of UCF201

Upon comparison of UCF201's original IC_{50} to its' IC_{50} following three seven-day rounds of treatment, an 11.28 fold increase was observed that is both worrisome and potentially insightful.

In order to demonstrate the mechanism of resistance to UCF201 was not due to similar mechanisms for known antimalarials, the intermittent resistant Dd2 line was also assayed for standard inhibitor IC_{50}s. The standard inhibitors used included CQ, MQ, ART and PYR. The standard inhibitor data demonstrated that there was no significant increase in the parasite IC_{50} for

the known antimalarials used, thus indicating that the specific mechanism of resistance to UCF201 is dissimilar to the mechanisms reported for CQ, MQ, ART and PYR.

The downside to this dramatic increase in parasite IC_{50} is the indication of an extremely high potential for resistance development to this specific compound. However, studies have shown that certain multi-drug resistant strains, such as the Dd2 that was employed for this study, have a higher propensity to develop drug resistance than other strains with no prior drug insensitivities (Rathod, McErlean et al. 1997). In fact, parasite phenotypes that have consistently developed resistance to multiple drugs are commonly referred to as ARMD (accelerated resistance to multiple drugs) phenotypes (Rathod, McErlean et al. 1997). Moreover, relative propensities for drug resistance development to known antimalarials has been shown to be increased by an increase in selective pressure of a given antimalarial compound (Nzila, Nduati et al. 2000). In light of this fact, the high selective pressure of 5X IC_{90} that was employed for the intermittent strain could also be a contributing factor to the extremely rapid resistance development that was observed. In future studies regarding resistance emergence to UCF201, it will be beneficial to use a parasite phenotype that is sensitive to current antimalarials (such as *P.falciparum* 3D7). If a sensitive parasite strain is used, it is probable that rapid resistance emergence will not occur, and the results obtained will be more applicable to the most prevalent malarial infections.

However, the resistance seen could also lead to the elucidation of UCF201's specific target. This could be accomplished by comparing the previously sequenced Dd2 genome to that of the generated resistant Dd2 parasite. If there are any substantial genomic differences, the afflicted genes and proteins they encode can be determined via the *Plasmodium* genomic

database known as PlasmoDB. Identification of the gene could potentially lead to identification of the drug's target. Some may question the practicality of sequencing an entire genome for these purposes. However, our lab has recently consulted with the Genomics Division Director at the University of Florida, Dr. D. M. Amador, and he has deemed the task practical via the following assumptions: ten parasite isolates/stages are provided and two different post-Sanger sequencing platforms are used (he suggests Roche 454 Titanium XL and Illumina GAIIx platforms). The only factor that could be an issue is the cost of such sequencing, but, even so, the data that can be obtained from such projects is well worth the capital.

However, other methods can be used to identify the drug's specific target if genomic sequencing is not performed or if the results are inconclusive. These secondary approaches utilize the same concepts that govern immunoprecipitation (IP) techniques. Essentially, the compound of interest can be tagged with a compatible tag in such a way that the activity of the compound is not disrupted. This can be confirmed by comparing the tagged drugs parasite IC_{50} to that of the untagged drug. Upon validation of similar IC_{50}s, the tagged compound can then be administered to the various stages of the parasites erythrocytic life cycle. These cells can then be lysed in order to obtain a crude lysate that contains all parasite proteins of the various stages along with the specific protein-drug (tagged) complex. This crude lysate can then be run over an affinity matrix that is specific for the given compounds tag. The affinity matrix will specifically interact with the drug-protein complex and effectively pull-down the drug's target protein. An alternative approach is the immobilization of the drug onto an affinity matrix such as dextran or agarose beads. Following the drugs immobilization, the crude parasite lysate (with all parasitic proteins and protein complexes) can then be administered to this compound affinity matrix to

allow the protein(s) within the lysate to specifically interact with drug on the affinity column. Following this, the non-interacting proteins can be washed away, and the specific protein that interacts with the compound of interest can be eluted by applying a gradient of the target compound. The eluted protein(s) can then be analyzed to identify the drug's specific molecular target(s).

It should also be noted that this resistance development data could also be inaccurate. Although the assays (n=3) which were performed prior to the drug treatment are completely reliable, the subsequent screening was limited to n=2 after only the third round of treatment. These limitations were a reality because of the underlying limitations in compound availability. However, once more compound is synthesized and obtained it will be possible to validate these conclusions as well as potentially discover further resistance development that occurred after this experiment was performed.

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