ASSESSING ANTIMALARIAL CIDALITY IN Plasmodium falciparum Parasites

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ABSTRACT

The rise of resistance to commonly used antimalarial medications, such as chloroquine, has exacerbated a global health burden. Years of study of chloroquine resistance has focused on low concentrations of drug that result in an inhibition of parasite growth, known as cytostatic activity. In a clinical setting, the concentration of chloroquine peaks at much higher concentrations and kills parasites. Defined as “cytocidal” activity, the ability of higher concentrations of antimalarials to kill parasites has only recently been explored. Comparing in vitro chemical models of hemozoin inhibition (the predicted mechanism of action of drugs like chloroquine) to the in vivo activities supports that there is a different target at cytocidal concentrations. To probe these possible targets, a novel activity-based protein profiling probe was synthesized, relying on copper-catalyzed azide-alkyne cycloaddition to conjugate a photoaffinity probe of chloroquine to biotin, for mass-spectroscopy proteomics, and fluorophores, for subcellular-localization by fluorescence microscopy.

In addition to understanding the cellular effects of cytocidal drug activity, the concept of cytocidality is integrated into the search for new combination therapies. High throughput screening of combinations of known antimalarials and compounds currently under clinical review identified a series of compounds that showed possible synergistic activities with known antimalarials. One class of these compounds was found to be phosphatidylinositol 3-phosphate kinase (PI3K) inhibitors, known to bind to human PI3K and effect its action in autophagy and...
other signaling pathways. Autophagy has only recently been explored in *Plasmodium falciparum*, and has been implicated in the cytocidal activity of chloroquine. Therefore, these PI3K inhibitors were paired with each of the drugs of the currently used therapy Coartem (artemether and lumefantrine), and their interaction was defined as synergistic, additive, or antagonistic under both the cytostatic and cytocidal conditions. Cytocidal, but not cystatic, synergy was observed when another PI3K inhibitor, GSK2126458, was paired with either artemether or lumefantrine in both chloroquine sensitive and chloroquine resistant strains. Understanding how drug activities change under cytostatic vs. cytocidal conditions will facilitate a better understanding of resistance and the identification of novel therapies.
This work is dedicated to my friends and family for their love and support,

 but especially to my mom, Mary,
 who always knew that I would make it here, even when I did not.

 Many thanks and much love,
 Katy S. Sherlach
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LIST OF ABBREVIATIONS

AAzCQ- alkynylated azido-chloroquine
ABPP- activity-based protein profiling
ACN- acetonitrile
ACT – artemisinin combination therapy
AQ – amodiaquine
ATM- artemether
ART – artemisinin
ATG- autophagy-related protein
ATP- adenosine triphosphate
ATSU- artusenate
AzBCQ- azido biotinylated chloroquine
BHIA – β-hematin inhibitory activity
CAR – cellular accumulation ratio
CD – cinchonidine
CDC – Centers for Disease Control and Prevention
CN – cinchonine
CM- complete media
CQ – chloroquine
CQR – chloroquine resistant or chloroquine resistance
CQS – chloroquine sensitive or chloroquine sensitivity
CT- Chou-Talalay method of defining drug interaction
CuAAC - copper-catalyzed azide-alkyne cycloaddition
DAG – diacylglycerol
DG – 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylcholine
DHLA – dihydrolipoic acid
DIAD – diisopropyl azodicarboxylate
DIC- differential interference contrast
DMAP- 4-dimethylaminopyridine
DMSO – dimethyl sulphoxide
DNA- deoxyribonucleic acid
DRAMPs- drug resistance associated membrane proteins
DT – 1,2-dioctanoyl-sn-glycerol-3-phosphatidylcholine
DV – digestive vacuole
DVM – digestive vacuolar membrane
EPA- United States Environmental Protection Agency
eQD – 9-epi-quinidine
eQN – 9-epi-quinine
ESI – electrospray ionization
EtOH- ethanol
FIC – fractional inhibitory concentration
$\text{FIC}_{\text{index}}$ – fractional inhibitory concentration index, or combination index
FLD – fractional lethal dose
$\text{FIC}_{\text{index}}$ – fractional lethal dose index, or combination index
FPiX – ferrriprotoporphyrin IX

FT – Fourier transform

GCMS- gas chromatography mass spectroscopy

GSK212- GSK2126458

Hb – hemoglobin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV/AIDS- human immunodeficiency virus/acquired immune deficiency syndrome

HPLC- high performance liquid chromatography

Hr(s)- hour(s)

Hs- Homo sapiens

Ht – % hematocrit

H-T – head-to-tail dimer

huMDR1- Human Multidrug Resistance Protein 1

Hz – hemozoin

IARC- International Agency for Research on Cancer

IC$_{50}$ – 50% growth inhibitory concentration

IM- incomplete media

iRBC- infected red blood cell

LA – lipoic acid

LD$_{50}$ – 50% lethal dose

LF- lumefantrine

LiOH- lithium hydroxide
mA- milliamperes
MAG – monoacylglycerol
MDR- multi-drug resistance
MDR1- human multi-drug resistance transporter
MeOH – methanol
MES – 2-(N-morpholino)ethanesulfonic acid
metHb – methemoglobin
Min(s)- minute(s)
MOA- mechanism of action
MQ – mefloquine
MS – mass spectrometry
mTOR- mammalian target of rapamycin
MW – molecular weight
m/z – mass-to-charge ratio
NaOH- sodium hydroxide
NCATS- National Center for Advancing Translational Science
NIH- National Institutes of Health
NMR – nuclear magnetic resonance
NVP- NVP BGT228
%p – % parasitemia
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline
PBS-T - phosphate buffered saline with Tween 20
PC - phosphatidylcholine
PCE - perchoroethylene
P. falciparum/Pf - Plasmodium falciparum
PfATG8 - Plasmodium falciparum autophagy-related protein 8
PfCRT - Plasmodium falciparum chloroquine resistance transporter
pfcrt - Plasmodium falciparum chloroquine resistance transporter gene
PfMDR1 - Plasmodium falciparum multi-drug resistance transporter
PfNHE - Plasmodium falciparum sodium-proton exchanger
PHIL - Public Health Image Library
PI3P - phosphotidylinositol 3-phosphate
PI3K - phosphotidylinositol 3-kinase
pMAG - 1-monopalmitin
PNBA - para-nitrobenzoic acid
PVM - parasitophorous vacuolar membrane
QD - quinidine
QN - quinine
QNR - quinine resistant or quinine resistance
QNS - quinine sensitive or quinine sensitivity
QTL - quantitative trait loci
RBC - red blood cell
Rf - resistance factor
SDCM- spinning disk confocal microscopy
SDS – sodium dodecyl sulfate
SEM – standard error of the mean
SM- starvation media
sMAG – 1-monostearin
TAG – triacylglycerol
TAMRA- tetramethylrhodamine
TgATG8- Toxoplasma gondii autophagy-related protein 8
THF- tetrahyrdofuran
TLC – thin layer chromatography
Tris – tris(hydroxymethyl)methylamine
UV – ultraviolet
V- volts
v/v – volume per volume
VAR – vacuolar accumulation ratio
VIS – visible
WHO – World Health Organization
w/v – weight per volume
VPL- verapamil
Vps34- vacuolar protein sorting 34 protein
ZnPIX – zinc(II)protoporphyrin IX
CHAPTER I

INTRODUCTION

1.1. General Malaria Background

Malaria is a disease of global importance. Across the globe, 3.3 billion people (roughly half the world’s population) live in malaria-endemic areas spread throughout 104 countries. Among those 104 countries, 35 account for 98% of all deaths due to the disease (30 of these are in sub-Saharan Africa) (Centers for Disease Control. Malaria Facts). In 2010 alone, the World Health Organization (WHO) recorded over 210 million cases of malaria, leading to nearly 655,000 deaths. Malaria is the cause of 20% of all childhood deaths in Africa (World Health Organization. World Malaria Report 2010). Despite global eradication efforts, malaria remains the 5th leading cause of death from infectious disease worldwide, and the second-leading cause of death from infectious disease in Africa, behind HIV/AIDS (Centers for Disease Control. Malaria Facts).

Malaria is caused by parasitic infection of one of 5 or 6 species of *Plasmodia: malariae, ovale, vivax, knowlesi* (zoonotic), and *falciparum* (which is responsible for the most severe cases and the most mortality) (Centers for Disease Control. Malaria Disease). Recent reports have identified a possible second zoonotic infection of a human with *P. cynomolgi* (Ta et al. 2014). Malaria infections can range from uncomplicated, where the patient experiences a two-day cycle of fevers, chills, vomiting and headaches, to severe cases. In a severe case of malaria, the repeated lysing of erythrocytes can lead to severe anemia, along with seizures, low blood pressure, kidney failure, coma, and death (Centers for Disease Control. Malaria Disease).
The *Plasmodium* parasite has 2 hosts: humans, and the female *Anopheles* mosquito vector. Throughout the two hosts, the parasite transforms from sexually-reproducing forms (mosquito), and asexually-reproducing forms (human), as shown in Figure 1.1. When the female mosquito bites a human to consume a blood meal, sporozoites are injected into the blood stream. The sporozoite invades a hepatocyte in the liver. During the liver infection, a single sporozoite can reproduce into 30,000-40,000 daughter merozoites. The asexually-reproducing form then moves into the blood stream, infecting host erythrocytes. The red-blood cell (RBC) stage is responsible for the symptomatic phase of the disease. Over the course of the 48-hour life cycle, the merozoite invades a red-blood cell, consumes host hemoglobin during the trophozoite stage, and replicates in schizogony to form new merozoites that burst from the erythrocyte to continue the next life cycle (Centers for Disease Control. Malaria Facts; Centers for Disease Control. Malaria Biology). During the asexual blood-stage reproduction, the parasite will also occasionally form a sexually-segregated gametocyte, which, when consumed by another mosquito, can begin sexual reproduction and produce sporozoites within 10-18 days (Centers for Disease Control. Malaria Biology).
1.2. Hemozoin and Quinoline Antimalarials

The symptomatic phase of malaria, which is the predominant target of chemotherapeutics, is the RBC stage. Common antimalarials, including quinolines and artemisinin, are shown in Scheme 1.1.
Scheme 1.1. Structures of Known Antimalarials. Reprinted with permission from Gorka et al. 2013c. Copyright 2013 American Chemical Society

As the parasite grows and moves through its intraerythrocytic life stages, ring, trophozoite, and schizont, the host hemoglobin is ingested and trafficked to the digestive vacuole (DV), as seen in Figure 1.2. The DV is a lysosome-like organelle where proteases digest hemoglobin (Hb), releasing toxic ferrirprotoporphoryin IX (FPIX). Approximately 85% of the RBC Hb is digested by the trophozoite (Gligorijevic et al. 2006a). FPIX release can result in lipid peroxidation and disruption of membrane integrity. In order to avoid the deleterious effects of free FPIX, the parasite sequesters the free heme as crystalline hemozoin (Hz). Hemozoin’s unit cell is comprised of head-to-tail dimers of the FPIX. In a head-to-tail dimer, the iron center
of one of the two heme units is coordinated to the carboxylate of the other, and vice versa. The precise mechanism of hemozoin formation is not fully understood, but probably involves additional heme species such as monomer, amorphous aggregate, and μ-oxo dimer (Gorka et al. 2013c). It is believed that the first step involves the aggregation of FPIX under acidic conditions. Ionization of the central iron, and removal of the axial ligand alter the self-association of FPIX. In the pH 5.5 conditions of the DV, this self-associated species has altered sedimentary behavior compared to both neutral pH and more highly acidic environments (Crespo, et al. 2010).

Figure 1.2. Diagram of an Infected Erythrocyte and Chloroquine Distribution. Within the red-blood cell, the parasite resides within a parasitophorous vacuole (light brown) and its own cell membrane (dark brown). Host hemoglobin is consumed by the parasite and digested within the digestive vacuole (blue), forming the malaria pigment hemozoin (black). Transport proteins like PfCRT could reduce accumulation of CQ in the DV.
Due to their weak-base nature, some antimalarial compounds, particularly quinolines such as quinine and chloroquine, will accumulate in the DV by acid trapping. Nanomolar levels of external CQ can accumulate within the DV to millimolar levels (Chong, et al. 2003). The proposed mechanism of action of these compounds involves interrupting the hemozoin formation process, releasing toxic FPIX (Gorka et al. 2013c). Examination of hemozoin formation in live parasites can be performed through the use of differential interfering contrast on a spinning-disk confocal microscope. Treatment of infected RBCs with twice their 50% inhibition concentration (2xIC$_{50}$) concentrations of CQ resulted in a reduction of hemozoin by 30%, while the parasite continued to progress normally through the cell cycle (Gligorijevic et al. 2006a). It is possible that the quinolines interrupt hemozoin formation by incorporating into the crystal face to block further growth, or by sequestering pre-crystalline forms away from the growing crystal (Chong, et al. 2003). Studies of the pH-dependent solubility of FPIX show a change in the titration curve in the presence of quinoline antimalarials. The concentration of the quinolines in this aggregate form is not stoichiometric, and suggests a novel, still yet undefined, nucleation phenomenon, which could impact the crystallization pathway (Ursos, et al. 2001).

Another technique used to examine quinoline-FPIX interactions that could interfere with hemozoin formation is nuclear magnetic resonance (NMR) spectroscopy. Studies have shown that CQ, quinine, and quinidine can form π-complexes with μ-oxo dimers, while CQ can also form a dative complex with monomer FPIX (Leed et al. 2002; de Dios et al. 2003). When quinolines bind, they can alter the pH-dependent equilibrium of the various forms of FPIX. Chloroquine binds to FPIX and promotes the μ-oxo form, while quinine promotes the formation of monomer (Casabianca et al. 2008). The formation of hemozoin is associated with lipid droplets inside the DV (Gorka et al. 2013c). μ-oxo dimer has been observed to partition into
detergent micelles, where CQ is not very soluble. It is possible, with the interaction of CQ and μ-oxo dimer, that the drug is able to remove μ-oxo dimer from lipid environments and thereby slow the crystallization (Casabianca et al. 2009). Quinine forms a complex with monomeric FPIX that is stabilized by hydrogen bonds and ion-dipole interactions. This complex can form an insoluble, non-crystalline aggregate that could prevent the proper crystallization of hemozoin (Alumasa et al. 2011).

*In vitro* analysis of the growth of β-hematin (a synthetic version of hemozoin) has been used to predict the activity of potential antimalarial drugs. Various systems have been used, ranging from acidic aqueous solutions, with more biological relevance, to those run in high levels of organic solvent, which are less applicable to the conditions of the DV. Histidine-rich proteins and lipids were shown to accelerate the formation of crystals when added to the assay (Egan 2008). However, if *in vitro* studies are meant to model *in vivo* activity, the assays would need to be biologically-relevant as far as solution composition, pH, temperature, and the presence of catalysts.

### 1.3. Chloroquine Resistance

After centuries of use of the naturally-occurring quinoline quinine, the synthetic 4,7-quinoline chloroquine was discovered in 1934, with wide-spread use beginning after the end of World War II. Chloroquine (CQ) was inexpensive, with a full course of treatment costing around $0.10, and effective, leading to a drop in mortality and the hope of eradication. Using CQ extensively in this effort resulted in the rise of resistant strains of *P. falciparum* initially along the Thai-Cambodian border in the late 1950s. Through the 1960s and 70s, resistance spread throughout Southeast Asia, India, and South America. Resistance in Africa was initially seen in
Kenya and Tanzania in the 1970s, and spread across the continent within a decade, as shown in Figure 1.3. The rise of resistance lead to a dramatic increase in mortality and morbidity, rendering CQ ineffective in many areas of the world (Wellems et al. 2001, Hartl 2004).

![Map of the Origin and Spread of Resistance](image)

**Figure 1.3.** Map of the Origin and Spread of Resistance. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Hartl 2004), copyright (2004).

### 1.4. Chloroquine Resistance and PfCRT

In order to explore the possible causes of resistance, the progeny generated from a cross between CQ sensitive (CQS) HB3 and CQ resistant (CQR) Dd2 was analyzed by quantitative trait loci (QTL) analysis of the resistance phenotype. The possible resistance-related gene was initially identified within a 36 kilobase pair region on chromosome 7; looking within that region
yielded *pfcrt*, where a series of mutations segregated with drug-response phenotype (Wellems *et al.* 1991; Fidock *et al.* 2000). *Pfcrt* encodes the protein Plasmodium falciparum chloroquine resistance transporter (PfCRT), which is localized to the DV membrane. Resistance was seen to require a series of genetic mutations in the gene *pfcrt*, in contrast to pyrimethamine resistance, which requires a single mutation in a gene (Wellems *et al.* 2001). Transfecting a sensitive line with “resistant” isoforms of the genes resulted in reduced susceptibility, confirming the role of PfCRT in resistance (Sidhu *et al.* 2002). Additionally, when a CQS strain, Sudan 106/1, that harbors 6 out of the 7 common Old World CQR mutations (missing K76T) was pressured with CQ, generation of alternative mutations at the same location in PfCRT (K76N, K76I) result in a decrease in susceptibility (Cooper *et al.* 2002).

The DV-localization of PfCRT suggests that the protein may transport CQ, moving it away from its target. Heterologous expression of PfCRT in yeast allowed for closer examination of its interaction with CQ, where it was shown to bind with a $K_D$ of around 400 nM (Zhang *et al.* 2004). Similarly, both heterologously-expressed and native PfCRT were labeled by a photoaffinity-analog of CQ, Azido-Biotinylated Chloroquine (AzBCQ). Successful competition of the probe with “cold”, underivitized, CQ, supported an overlapping binding site which was then explored by trypsin digestion and mass-spectroscopic analysis (Lekostaj *et al.* 2008). The presence of extracellular CQ resulting in the increased transport of PfCRT expressed in *Dictyoselium discoideum* suggested that trans-stimulated efflux may reduce the CQ present in the DV, removing it from its target. (Sanchez *et al.* 2007). Studies involving a fluorescent analog of chloroquine, NBD-CQ, and proteoliposomes containing purified PfCRT, were performed in order to determine a model of transport. In this study, trans-stimulation was not observed. The model proposed was that PfCRT acts like a channel, allowing the movement of CQ down an
electrochemical gradient, with more efficient transport for CQR parasites compared to CQS (Paguio et al. 2009). In support of this theory, decreased accumulation of $[^3$H]CQ was observed in CQR parasites under low nanomolar concentrations of extracellular CQ (Roepe 2011). In some studies, other anti-malarials and resistance-reversing compounds were hypothesized to be transported by PfCRT (Lehane et al. 2011).

In addition to altered drug transport, studies have observed effects of mutant PfCRT on the pH of the DV. Lehane and Kirk propose that protons are removed from the DV through the efflux of protonated CQ, termed a “proton leak” (Lehane et al. 2008). Bennet, et al., however, observed a difference in steady state pH of the resistant strains. In CQS strains, the DV pH is at 5.5; in CQR strains, this value decreases to 5.2 (Bennet et al. 2004a). Alteration in the pH of the DV can impact the pH-related solubility of FPIX isoforms, and therefore the availability of the proposed target of quinoline drugs.

In addition to PfCRT, another protein has been associated with CQR, PfMDR1. PfMDR1, when heterologously-expressed, binds the AzBCQ photoaffinity-analog of CQ (Pleeter et al. 2010). Although PfMDR1 does not segregate with CQR like PfCRT, it is possible that it modulates the accumulation of drug within the DV. Rorbach et al. (2006) proposed that it can import solutes into the DV, including drugs like CQ. Transfection and drug-pressure studies with PfCRT recapitulate only 70-90% of the 50% growth inhibitory concentration (IC$_{50}$) values of naturally-occurring resistant strains, suggesting that resistance could be multifactorial, although the lower values could be also due to lower levels of expression compared to the isoform’s parent strain (Waller et al. 2003; Roepe 2011). The involvement of other proteins, such as PfMDR1, is expected.
1.5. Cytostatic vs. Cytocidal Activity

Definition of PfCRT as the major cause of CQR relies on the effect on growth of culture in the continuous presence of low nanomolar levels of drug, resulting in definition of a shifted growth-inhibitory concentration, or IC$_{50}$ value. However, clinically relevant concentrations of CQ often reach the micromolar range (Salako et al. 1987; Khalil et al. 2011). Aside from one study of the effects of deferoxamine on *P. falciparum* (Whitehead et al. 1990), the ability of a drug to kill parasites has not been routinely quantified. Resistance could be due to a more complicated resistance mechanism than just altered accumulation in the consistent presence of low levels of drug. Studies of the stage-specificity of CQ show activity and resistance at every intraerythrocytic life stage, even those where hemozoin is not being formed (Gligorijevic et al. 2008). Additionally, CQ-treated parasites showed a reduced multiplicity of daughter nuclei during schizogony, as well as a reduced viability of parasites in the following cell cycle. These observations are not consistent with hemozoin formation being the sole target of CQ treatment (Gligorijevic et al. 2008). Further studies of CQ accumulation showed that when incubated with several different concentrations of external CQ, CQR parasites can accumulate CQ within their DVs to levels above that of CQS parasites while still retaining their viability. This study by Cabrera et al. (2009) showed that reduced accumulation in the DV resulting in reduced accessibility to target is not the sole cause of resistance.

In order to quantify the effects of high-concentration (cytocidal) bolus doses of drug, new assays were developed. In 2011, Paguio et al. modified the previously-used SYBR Green growth inhibition assay to wash drugs away after a set incubation time and monitor growth into the following cell cycle, generating the 50% cytocidal dose (LD$_{50}$). Examination of resistance to these bolus doses resulted in altered trends when contrasted with cytostatic activity defined by
The ten-fold increase of IC$_{50}$ values between CQS and CQR strains that is seen in cytostatic growth assays becomes 100-fold under bolus dose, cytocidal conditions. Relatedly, verapamil, which has been shown to chemosensitize certain strains of resistance parasites at cytostatic concentrations of CQ, has no effect on the cytocidal activity (Paguio et al. 2011). Another cytocidal assay that has been developed utilizes transfection with an overexpressed luciferase. Reduction in the generation of bioluminescence over an eight-hour incubation is determined to be due to cell death, allowing for examination of cell stage-specific effects and examination of the rate of kill for different drugs (Hasenkamp et al. 2013).

As mentioned earlier, transfection of CQS strains with CQR isoforms of PfCRT was shown to raise the IC$_{50}$ values to 70-80% of that seen in CQR strains (Waller et al. 2003; Roepe 2011). However, when CQR isoforms of PfCRT are transfected into a CQS background and examined for the resulting cytocidal activity, only 7-23% of the parent strain LD$_{50}$ is recapitulated (Gaviria et al. 2013). While the 20-30% reduction in IC$_{50}$ at the cytostatic level could conceivably be attributed to differences in expression level due to the transfection process, that cannot be the reason behind such a low value of LD$_{50}$ compared to the parent strain. Where the PfCRT isoform dictates the majority of cytostatic resistance, it does not contribute nearly as significantly to cytocidal resistance. Most likely, there is another process or other targets of CQ that are altered in CQR parasites that result in the high LD$_{50}$ values observed (Gaviria et al. 2013).

QTL analysis of a cross between CQS HB3 and CQR Dd2 parasite strains at the cytostatic level (using IC$_{50}$ data) led to the identification of pfcrt as the predominant source of resistance, with added contribution from a locus on chromosome 5 that includes the gene for PfMDR1. Repeating this process at the with LD$_{50}$ data, however, does not yield the same results.
The section of chromosome 7 that includes *pfcr* is detected, but at a lower significance than in the cytostatic analysis. The PfMDR1 locus on chromosome 5 was not detected in this screen. Additional contributions to cytocidal resistance were identified on chromosomes 6 and 8, with additive interactions between the two loci. Within these loci are genes that code for proteins involved in vesicle traffic, proteosome function, and lipid metabolism. The QTL analysis supports the hypothesis that the classical drug mechanism of hemozoin inhibition in the DV with reduced DV-levels of drug leading to resistance is not as applicable at the cytocidal level (Gaviria *et al.* 2013).

### 1.6 Autophagy in *Plasmodia*

These shifts in LD$_{50}$ data for drug resistant parasites indicate resistance to parasite cell death (not merely resistance to drug induced growth inhibition). The mechanism of drug-induced death in the malaria parasite has been debated. The identification of metacaspases encoded in the *Plasmodium* genome, along with observed disruption of mitochondrial electrochemical potential, DNA fragmentation, chromatin condensation, and externalization of phosphotidylserine by some drugs led some to propose that the parasite can undergo a modified version of apoptosis (Meslin *et al.* 2007; Deponte *et al.* 2004). However this is disputed by others who did not observe features of apoptosis in drug-treated parasites (Nyakeriga *et al.* 2006; Totino *et al.* 2008). Ultrastructural analysis of parasites showed no chromatin condensation, a nuclear morphology inconsistent with apoptosis. Any DNA fragmentation that was observed was seen in very few parasites, and it is possible that any apoptotic death may only account for a small percentage of the bulk population (Totino *et al.* 2008). In the cytocidal QTL analysis, no genes involved in the apoptosis programmed cell death pathway were observed within the
identified genomic regions, suggesting that the parasite does not die by that process (Gaviria et al. 2013). Additionally, the tell-tale signs of necrosis: swelling, disruption of the plasma membrane, precipitation of proteins, and nuclear dissolution, were not present. What has been observed following drug treatment, however, is vacuolarization of the cytoplasm suggesting another pathway of cell death, autophagy (Totino et al. 2008, Gaviria et al. 2013).

Autophagy, or “self-eating”, is a cellular process where, during periods of nutrient deprivation, a cell can recycle biomolecules through degradation in lysosomes, as well as breaking down damaged molecules and organelles. A survival mechanism in periods of starvation, autophagy has also been designated as a method of programmed cell death, alongside apoptosis and necrosis. Over 30 autophagy-related (ATG) proteins have been defined, initially in S. cervisiae yeast. Typically, autophagy is present only at a low constitutive level. The mammalian target of rapamycin (mTOR) initiates the autophagic process when activated. Downstream, the ATG1 complex begins the formation of a perivacuolar structure known as the phagophore, the proposed nucleation site for vesicle formation. Nucleation of the phagophore requires enrichment of the membrane with phosphotidylinositol 3-phosphate (PI3P) by a class III phosphotidylinositol 3-kinase (PI3K) called VPS34. The phagophore is enlarged through the generation of lipidated ATG8 by an ATG7/ATG3 complex, which surrounds the selected cargo. The completed phagophore, with lipidated ATG8 on the surface, then fuses with the lysosome or vacuole (Brennard et al. 2011).

While well-defined in higher eukaryotes and yeast, autophagy in early-diverging parasitic protists like Plasmodia had only recently been explored. Despite the lack of a clear mTOR homolog in the Plasmodium genome, there are signs that the cascade does exist (Sinai and Roepe 2012). The metamorphosis between life cycle stages of the parasite suggest that autophagy is
active in some form in the parasite. Additionally, treatment of parasites with 3-methyladenine (3-MA), a VPS34 inhibitor, slows sporozoite differentiation (Brennand et al. 2011). Genomic analysis suggests the presence of a VPS34 ortholog, along with homologs of other ATG proteins (Brennand et al. 2011). The lipid essential for the generation of the phagophore, PI3P, has been found to be present in the membranes of the DV as well as the plastid apicoplast, and in single-membraned vesicles (Tawk et al. 2010).

Another ATG protein homolog that has been identified is PfATG8, which has been determined to be essential for survival of the parasite, and may have other functions in the cell besides just in the autophagy cascade (Brennand et al. 2011). Intraerythrocytic parasites, as well as liver-stage parasites show localization of GFP and mCherry-conjugated ATG8 to the apicoplast, with no vesicles observed (Kitamura et al. 2012; Eickel et al. 2013). However, later studies demonstrated the presence of ATG8-positive vesicles. Tomlins et al. identified vesicles that co-localize with RAB7, a known autophagy regulator in the periphery of the DV. The same study showed PfATG8 present in both its lipidated and delipidated forms (Tomlins et al. 2013) Cervantes et al. observed PfATG8-positive vesicles, as well as apicoplast localization, throughout the intraerythrocytic life cycle as well as gametogenesis. Additionally, relocalization of the vesicles was observed under high doses of CQ, as well as bafilomycin A, but not under mild nutrient deprivation or artemisinin treatment (Cervantes et al. 2014). In the Roepe Lab, ATG8 puncta have been detected upon starvation, suggesting that these may be classic autophagic vesicles (Sinai and Roepe, 2012; Gaviria et al. 2013). Immunofluorescence microscopy of parasites labeled with an antibody generated against the ATG8 of the fellow Apicomplexan parasite Toxoplasma gondii show a punctate distribution indicative of active autophagy (Ghosh et al. 2012). A redistribution of ATG8 puncta in P. falciparum occurs after
cytocidal CQ treatment, but not cytostatic, and is altered in CQR parasites versus CQS, as shown in Figure 1.4 (Gaviria et al. 2013). While starvation of CQR parasites produces a redistribution phenomenon similar to that seen in CQS parasites, treatment with pharmacologically-equivalent amounts of CQ (2xLD₅₀) does not. Transfection of CQS with a CQR PfCRT isoform (C₄Dd2) produces a response in between that of the CQS and CQR strains. Western blot analysis of parasite extracts through utilization of the same anti-TgATG8 antibody shows the expected PfATG8 bands at 15 and 17 kDa, representing the lipidated and delipidated ATG8 forms (Gaviria et al. 2013).
Figure 1.4. Quantified PfATG8 Puncta Distribution for Synchronized Trophozoite Parasites. Shown are CQS HB3 (top) CQR Dd2 (middle) and transfectant C4\textsuperscript{Dd2} (bottom) under different conditions. Far left, “CM” = control culture conditions, far right, “SM” = iRBC in starvation media for 6 hr. In between are puncta quantified for iRBC treated for 6 hrs with the indicated [CQ]. Black bars in each panel denote 2×IC\textsubscript{50} [CQ] for the strain, hashed bars denote 2×LD\textsubscript{50} [CQ] for the strain. Data are the average of at least 20 iRBC, +/- s.d., and puncta that are ≥3.5 μm from DV hemozoin optical density are plotted. Reproduced from Gaviria et al. 2013. Open Access.
The presence of a single mitochondrion within the malaria parasite makes classical autophagy of mitochondria under starvation less desirable. The presence of possible vesicles containing ATG8 near Maurer’s clefts, external to the parasite, which have been proposed to be involved in vesicle traffic, suggests that the stimulation of autophagy in *Plasmodia* may result in increased uptake of host hemoglobin or in extracellular nutrients (Gaviria *et al.* 2013). The presence of lysosomotrophic compounds, like CQ, might then preclude the fusion of the autophagic/endocytotic vesicles with the DV (Gaviria *et al.* 2013). In fact, previous studies had shown an increase in the presence of hemoglobin-containing vesicles in the parasite following CQ treatment (Hoppe *et al.* 2004). Genetic comparisons of CQR and CQS genomes have discovered that 16 of 42 possible orthologs of ATG genes lay within regions identified by various drug resistance QTL analyses. Within these 16, looking for mutations that segregate with resistance phenotype leads to the PI3PK PfVPS34, which contains mutations in the calcium-binding region for CQR parasites. Since *P. falciparum* lack an mTOR ortholog, mutating VPS34 may have resulted in the altered autophagy cascade observed in resistant parasites (Gaviria *et al.* 2013).

**1.7. Drug Target Identification by Activity-Based Protein Profiling**

With a renewed focus on cytocidal drug treatment and the possible causes of cytocidal resistance, I propose that a technique known as Activity-Based Protein Profiling (ABPP) can be used to identify possible cytocidal drug targets in parasite cells. ABPP relies on covalent attachment to link small-molecule probes to their biomolecule targets, along with the ability to detect the covalently-linked product by such methods as radioactive isotope labeling, fluorescence, and affinity labels. The advent of high-throughput screening has facilitated the
development of new drugs, but techniques like ABPP are needed to elaborate the mechanism of action of a novel therapeutic, along with potential on and off-target interactions in whole cells or cell-lysates (Raghavan et al. 2009; Best 2009). ABPP has been used to investigate the activity of human cytochrome P450s, serine hydrolases, proteasomes, and cathepsin proteases, as well as the targets of phenyl sulfonates (Wright et al. 2007; Wright et al. 2009; Liu et al. 1999; Sletten et al. 2009; Speers et al. 2003).

ABPP requires generation of a probe that can form a covalent bond with the target of interest. An ABPP probe is comprised of 3 parts: the pharmacophore, the reactive cross-linker (if the drug is not an irreversible inhibitor), and a purification/visualization handle. The reactive cross-linkers are typically photo-reactive compounds such as aryl azides. UV-illumination of aryl azides results in the generation of N₂ and a reactive nitrene, which inserts into nearby C-H sigma bonds (Burdinski et al. 2006; Keana et al. 1990). In order to avoid ring-expansion reactions, which reduce the cross linking efficiency, aryl azides can be perfluoronated, resulting in approximately 45% bond insertion. These perfluoronated aryl azides do absorb in the same UV region as most biopolymers, but the differences in molar absorptivity result in the activation of the probe with minimal biomolecular degradation (Keana et al. 1990).

Labeling of the targets by ABPP probes is typically followed by proteome analysis techniques. Typically, proteomes are investigated either through gel-based (single or two-dimensional separation) or chromatography-based (HPLC or avidin-affinity) techniques. Following separation of the identified proteins, mass spectrometry can be used for identification of the trypsin digestion products of the target proteins (Salisbury et al. 2007). ABPP allows for selectivity in proteomic methods that is not necessarily possible in global methods. The use of
separation methods and detection of probe-labeled proteins reduces the background signal present from unrelated proteins, particularly those that may be in high abundance (Best 2009).

ABPP has been used previously to study drug binding in *P. falciparum*. Foley *et al.* utilized an iodine-125 labeled CQ analog, which labeled 2 proteins (42 and 33 kDa). However, the photoreactive group is directly attached to the aromatic pharmacophore, severely altering the electronic structure of the drug (Foley *et al.* 1994) and biasing drug-drug target binding. Lekostaj *et al.* utilized a biotinylated analog of CQ that also contained a perfluoroazido cross-linking group to study the binding of CQ to PfCRT, both endogenously and in heterologously-expressed and purified protein. The photoreactive group was attached to an aliphatic linker, preventing electronic interactions with the quinoline ring. Competition with underivitized CQ supported overlap of the binding site of the probe and the drug, and trypsin digestion and mass-spectroscopic analysis allowed for identification of the binding pocket (Lekostaj *et al.* 2008). A similar probe for artemisinin has also been synthesized (Barton *et al.* 2010). The reactive radical mechanism of artemisinin results in covalent attachment to its target. Coupling artemisinin (ART) to biotin creates a probe that could be used to identify *in vivo* targets (Barton *et al.* 2010).

Global proteomic analyses performed on *Plasmodium* include: comparison of resistance and sensitive strains (Koncarevic, *et al.* 2007), variations in protein levels across the various life-stages (Florens, *et al.* 2002), comparison of proteomes under ART and CQ pressure (Prieto, *et al.* 2008), and the oxidative damage caused by CQ (Rafar, *et al.* 2008). In theory, ABPP could be used to more directly probe the possible targets for CQ in a more selective manner than just surveying expression differences or oxidative damage.

Purification of the cross-linked target is typically achieved through conjugation to biotin, while conjugation to a fluorophore allows for visualization. These bulky reporter tags can
become an issue in competition experiments that examine the overlap of binding pockets, particularly since the added size of the cross-linking tag and purification handle can be roughly 50% of the size of the probe. This added steric bulk and altered hydrophobic/hydrophilic properties can alter the bioavailability and binding of the probe as compared to the drug of interest. One way to reduce the impact of added moieties is to split the probe into 2 parts to be coupled later through a bioorthogonal “click chemistry” reaction, as shown in Figure 1.5. “Click chemistry” is a term used for selective and high yielding reaction. Bioorthogonal click chemistry reactions include: the Staudinger Ligation, strain-promoted azide-alkyne cycloaddition, and copper-catalyzed azide-alkyne cycloaddition (CuAAC) (Salisbury et al. 2007; Raghavan et al. 2009).

Copper-catalyzed azide-alkyne cycloaddition (CuAAC), can be utilized in order to attach the purification handle after cross-linking by adding a terminal alkyne to the probe and an azide to the tag, or vice versa (Salisbury et al. 2007; Best 2009). CuAAC is a [3+2] cycloaddition reaction that converts a terminal alkyne and an aliphatic azide into a 1,4-substituted 1,2,3-triazole, as shown in Figure 1.6. The reaction proceeds through a 6-membered metallocycle, followed by ring contraction to produce the triazole product. Direct cycloaddition is disfavored by more than 20 kcal/mol. The presence of the copper(I) catalyst results in an increase in reaction rate by 7-8 orders of magnitude (Himo et al. 2005).
Figure 1.5. Integrating Bioorthogonal Chemistry into Activity Based Protein Profiling. A probe derivitized with a photoaffinity tag can be covalently linked to the target with a smaller handle than the traditional fluorophore or biotin tags used in single-molecule ABPP. Following covalent linkage, the small handle can be reacted with its partner in a bioorthogonal reaction, allowing for purification or visualization, depending on the desired detection system. Modified and reprinted with permission from Best 2009, Copyright 2009 American Chemical Society.

The reaction is tolerant of a wide range of conditions, including aqueous environments, as well as various temperatures, redox, and pH conditions. (Himo et al. 2005) Since copper(I) is not stable in air, a copper(II) source is typically used, along with a reductant like sodium ascorbate, to produce copper(I) in situ (Hong et al. 2009). Toxicity of copper(II), as well as the production of reactive oxygen species by ascorbate, result in a reduced ability to use CuAAC in live cells (Salisbury et al. 2007; Best 2009; Hong et al. 2009). However, recent work has examined the reduction of copper toxicity through the use of chelating ligands (Gupta et al. 2005; del Amo et al. 2010; Kennedy et al. 2011). Likewise, if CuAAC is used after fixing and permeabilizing a cell, the toxicity of copper(I) is no longer problematic (Best 2009), and can be used to facilitate investigation of the effects of drug treatment on Plasmodium parasites.
Figure 1.6. CuAAC Reaction Scheme. A terminal alkyne forms an activated copper acetylide (1), that is attacked by the first azide nitrogen (2). Formation of a 6-membered metallocycle (3), is followed by contraction of the ring (4), and release of the copper catalyst, yielding the triazole product (5) (Himo et al. 2005).

1.8. Drug Combination Analysis

The rise of resistance to many forms of malaria treatment, from CQ and fellow quinolines like QN, to the combination therapy of sulfadoxine and pyrimethamine, has lead to the discovery of new antimalarials and a renewed focus on novel combination drug therapies. The current front line of malaria therapy is ART combination therapy (ACT). ART is a natural product that was isolated in the 1970s with a reactive endoperoxide that undergoes a rapid onset of action. Optimization of the drug for solubility led to the generation of a series of related compounds including artesunate (ATSU), artemether (ATM), and dihydroartemisinin (DHA) (Olliaro et al.)
Artemisinins are rapidly cleared \textit{in vivo}. In formulating a ACT combination, the artemisinin derivative is typically paired with a partner drug with a longer half-life, to ensure full clearance of the infection. When ATM was partnered with the pseudo-quinoline lumefantrine (LF), also shown in Scheme 1.1, preclinical studies reported synergistic interactions between the two drugs. These studies are not available outside China and are not described in any level of detail (Olliaro \textit{et al.} 1995). When ATM is administered, it is rapidly taken up and partially metabolized into DHA, which both have a half-life of about 1 hour. LF, which is absorbed much more slowly, is also eliminated slowly; its half-life is between 3 and 6 days. A fixed ratio of 1:6 ATM:LF was observed to be the optimum combination to administer, which is now used under the name “Coartem”, and resulted with higher parasite clearance compared to monotherapy of either drug (White \textit{et al.} 1999; Novartis Pharmaceuticals Corporation. CoArtem Product Information).

The available reports of ART-LF synergy trace back to a study by Alin \textit{et al.} in 1999 that utilized a commonly-used technique known as checkerboard analysis to quantify the interaction via a probit method of data analysis. Examining two strains, T-996 and LS-21, the authors noted synergy at the IC$_{50}$, IC$_{90}$, and IC$_{99}$ levels, by converting growth data into quantile units. The determination of synergy is based on predicted growth of the two compounds acting independently (derivation not discussed) (Alin \textit{et al.} 1999). Additionally, a study in 2007 by Thriemer \textit{et al.} identified synergy between LF and the ATM metabolite DHA at their IC$_{50}$ values utilizing the isobologram method, and Wong \textit{et al.} (2011) identified slight synergy between DHA and the LF metabolite desbutyl-lumefantrine utilizing the same technique at IC$_{50}$ values.

Reports of ART tolerance in the Thai-Cambodian border region (Dondorp \textit{et al.} 2009) and the generation of temporary tolerance to LF \textit{in vitro} (Mwai \textit{et al.} 2012) suggest that novel
drug combinations should be explored before Coartem loses efficacy. Techniques to identify synergistic partner drugs, as mentioned above, include altering the concentrations and relative ratios of drugs through a checkerboard pattern on the assay plate, as well as fixed-ratio serial dilutions. Either technique can be used to generate the plot used to identify the interaction, known as an isobologram. Isobologram analysis requires comparison of the growth curve data of the drug in the presence of its partner with its monotherapy activity, generating a ratio known as a fractional inhibitory concentration (FIC), which is calculated for each compound at a series of fixed ratios between the two. When these data are plotted, the results are typically either a straight line between (1,0) and (0,1), a concave curve, or a convex curve. If the plot is a straight line, the interaction is known as additive. When the curve is concave, and lies below the line that defines additivity, the interaction is synergistic. If the curve is convex, the interaction is antagonistic. Three sample curves are shown in Figure 1.7. More complex curves are possible, if the interaction depends on the ratio between the two compounds (Berenbaum 1978).

A clear definition of synergy vs. additivity vs. antagonism is needed to avoid confusion. For instance, if the presence of an inactive compound simply augments the activity of a drug, this is not synergy; it is potentiation. Synergy requires the presence of two active compounds. Additionally, the combined activity of drugs A and B being less than the monotherapies does not mean the combination is necessarily synergistic; such a result could be cause by additive or slight antagonistic interactions (Chou 2006). In addition to the isobolograms focused on the ATM and LF combination, other antimalarial combinations have been studied. These include atovaquone/proguanil, atovaquone/DHA, 9-epi-ART/ART, artemisitene/ART, 9-epi-ART/ATSU, artemisitene/ATSU (Fivelman et al. 2004; Suberu et al. 2013).
Figure 1.7. Example of Possible Drug Interactions on an Isobologram. Hypothetical drugs A and B are examined to calculate their FIC values at 5 fixed ratios, and then plotted and the shape of the curve identified as it compares to the mathematical additive line (dotted) that connects (1,0) and (0,1).

Generating a full isobologram requires the analysis of various ratios between the two drugs, and results in a large number of required assays. Derived from the Law of Mass Action, a simpler method of identifying drug interaction type was developed by Chou and Talalay (known as the Chou-Talalay Method [C-T]), which only examines one ratio at a time (Chou et al. 1984). The FICs for each drug are calculated for that ratio in the same way as one would for an isobologram. However, instead of plotting them on a graph, these values are summed to generate
a combination index, or fractional inhibitory concentration index (FIC index). Based on their calculations, Chou and Talalay define additivity when the FIC\textsubscript{index} equals 1. When the FIC\textsubscript{index} is less than 1, the interaction is synergistic; when it is greater than 1, the interaction is antagonistic (Chou et al. 1984). Synergy could potentially arise from inhibition of pathways that undergo downstream cross-talk, or perhaps through interacting with the same pathway in two different locations. The isobologram or C-T analysis only determines the nature of the interaction, not how it comes to be.

Although those cut-offs hold mathematically, experimental variance leads to alteration. The cut-offs used in experimental analysis can vary from synergy less than 0.5 and antagonism greater than 4 (Bell 2005), to just expanding the additive range to run from 1 to 2 (Matthews et al. 2013). The choice of cut-offs can be somewhat arbitrary (Bell 2005). The Chou-Talalay method has been used to investigate the interaction of artemisinin with other natural products isolated from ART tea (Suberu et al. 2013), the analysis of possible drug partners emetine/DHA (Matthews et al. 2013), tapsigargin/ART, tapsigargin/OZ227 (Abiodun et al. 2013), and the interaction of chalcone derivatives and ART (Bhattacharya et al. 2009).

Importantly, until recently all examination of drug combinations \textit{in vitro} focused on cytostatic conditions. Recent examination of combinations at both the cytostatic and cytocidal levels demonstrates the possibility of altered interaction, both between CQS and CQR strains, but also within the same strain at different levels (Gorka et al. 2013b). For instance, the combination of CQ and amodiaquine (AQ) in Dd2 parasites is additive at the cytostatic level, but highly antagonistic under cytocidal conditions. Differences like this reinforce the need to examine possible novel combination therapies at both levels to generate a complete description of the interaction (Gorka et al. 2013b).
1.9. Objectives

In order to understand CQR, particularly at the cytocidal level, it is important to understand the trends associated with resistance, as well as identifying the targets of CQ treatment. Investigating *in vitro* chemical models of the proposed mechanism of action of antimalarials will be performed. The ability of an antimalarial to inhibit the formation of a synthetic version of Hz known as β-hematin will be measured and compared to both the cytostatic and cytocidal activities. Similarly, the effect of changing the stereochemistry of QN derivatives on heme-interactions will be examined and compared to their activities at the static and cidal levels. Comparing *in vitro* chemical data to the *in vivo* activity can shed light on whether the conventional Hz-inhibition mechanism of action is applicable under cytocidal conditions.

In order to probe the possible targets of cytocidal chloroquine treatment, a photoaffinity-based ABPP probe will be synthesized that will allow for investigation of the subcellular localization of CQ at IC$_{50}$ and LD$_{50}$ doses, as well as the potential target proteins, through microscopy and mass spectrometry, respectively. Previously, a probe known as AzBCQ was synthesized and used to probe binding to PfCRT both in parasites as well as purified from heterologous expression in yeast. In order to add experimental flexibility, as well as reduce the size of the probe, the large biotin moiety of AzBCQ will be removed and replaced with a terminal alkyne that can be coupled to fluorophores or biotin, depending on the experiment. Coupling will be performed selectively and in high yields in an aqueous environment through the use of CuAAC. This new probe, alkynylated azido chloroquine (AAzCQ) will be used to probe purified protein labeling, as a method of comparing it to the previous probe.
In investigating cidality in *Plasmodium falciparum*, identifying the targets of one antimalarial that has suffered from the evolution of resistance in the field can be complimented by investigation of novel therapies that will be active against strains known to be sensitive and resistant to chloroquine. In order to prevent the rise of resistance to new therapies, the WHO recommends identification of combination therapies, such as ACTs. Identification of novel compounds that are active against malarial parasites is step one; these compounds must also be screened in combination with other compounds to properly define their interaction. Isobolograms and C-T are commonly-employed methods to identify combinations as synergistic, additive, or antagonistic. A high-throughput screen of compounds in the clinical trial process alongside known antimalarials, performed in collaboration with the National Center for Advancing Translational Science (NCATS), can generate isobologram data and provide leads for new combinations. These leads will be examined in further detail by C-T at both the cytostatic and cytocidal levels. In thinking down the pipeline to clinical use, combinations should be understood at their bolus, cidal levels, and their longer-lasting, lower level, cytostatic concentrations.

By examining the characteristics of cytocidal activity, probing the cytocidal targets, and exploring new combinations, more can be defined concerning what cidality is in *Plasmodium falciparum*, and how it impacts the search for new antimalarial therapies.
CHAPTER II
MATERIALS AND METHODS

2.1 Materials

Routine chemicals, media, and solvents were reagent grade or better, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Newark, DE), and used without further purification, unless otherwise noted. Sterile tissue culture treated, black, flat bottom and non-sterile, clear polystyrene 96-well plates, and all other laboratory plastics were purchased from Fisher Scientific (Newark, DE). Kieselgel 60 silica and flexible thin layer chromatography (TLC) plates were purchased from Selecto Scientific (Suwanee, GA).

Dimethyl sulfoxide-d6 (DMSO-d6), methanol-d4 (CD3OD), chloroform-d (CDCl3), and other deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), and used without further purification. SYBR Green I nucleic acid gel stain, and Alexa Fluor and tetramethylrhodamine (TAMRA) dyes conjugated to streptavidin or azides were purchased from Invitrogen (Eugene, OR). 1,4-Diaminobutane, Cinchonine (CN), and QN monohydrochloride dihydrate were purchased from Acros Organics (Geel, Belgium). AQ dihydrochloride dihydrate, CQ diphosphate, and QD hydrochloride monohydrate were purchased from Sigma-Aldrich (St. Louis, MO). Compounds for high-throughput screening were obtained from suppliers such as Tocris Bioscience, Selleck Chemicals, Santa Cruz Biotechnology and Sigma Aldrich. GSK212, NVP BGT226, Torin2, and PIK 93 were provided by the Thomas Laboratory at the National Center for Advancing Translational Science (NCATS) at the National Institutes of Health (NIH). Anti-TgATG8 antibodies as antisera, monoclonal antibodies, and purified IgG were provided by the Sinai Lab (University of Kentucky, Lexington, KY).
2.1.1 Parasite Strains

*Plasmodium falciparum* clone HB3 (Honduras, CQS), Dd2 (Indochina, CQR), and D10 (Papua New Guinea, CQS) were obtained from the Malaria Research and Reference Reagent Resource Center (Manassass, VA). *P. falciparum* line K76I, selected under CQ drug pressure of the CQS 106/1 (Sudan) clone (Cooper 2002), was a generous gift of Dr. Roland Cooper (Dominican University of California, San Rafael, CA).

2.2 Methods

2.2.1 General Spectroscopic Methods

NMR spectra were obtained on a Varian FT-NMR spectrometer with the following frequencies: $^1\text{H}$- 400 MHz, $^{13}\text{C}$- 100 MHz, $^{19}\text{F}$- 376 MHz. Spectra were obtained with either CDCl$_3$ or CD$_3$OD, as specified, with the residual solvent peak used as reference. ESI- mass spectra were collected in positive ion mode on a Varian 500MS mass spectrometer using LC-MS grade acetonitrile as a solvent. The electrospray needle was operated at 5 kV using N$_2$ as both the nebulizing gas (35 lb/in$^2$) and the drying gas (350 °C, 10 lb/in$^2$). Sample was delivered using a syringe pump employing a 1 mL syringe at a constant flow rate of 200 μL/min.

2.2.2 *Plasmodium falciparum* Culturing

Off-the-clot, heat-inactivated pooled type O$^+$ human serum and type O$^+$ human whole blood were purchased from Biochemed Services (Winchester, VA). Custom 5% O$_2$/5% CO$_2$/90% N$_2$ gas blend was purchased from Robert’s Oxygen (Rockville, MD).
All *P. falciparum* strains were maintained using the method of Trager and Jensen (Trager 1976) with minor modifications. Briefly, cultures were maintained under an atmosphere containing 5% CO$_2$, 5% O$_2$, and 90% N$_2$ gaseous mix at 2% hematocrit and 1-2% parasitemia in RPMI 1640 supplemented with 10% type O$^+$ human serum, 25 mM HEPES (pH 7.4), 23 mM NaHCO$_3$, 11 mM glucose, 0.75 mM hypoxanthine, and 20 μg/L gentamicin, with regular media changes every 48 h. Culture health was verified through smearing 3 μL of culture onto a glass slide. Following fixation in methanol for 3 minutes, slides were stained with Giemsa Stain (Sigma Aldrich, St. Louis, MO). Giemsa-stained smears would also be counted to calculate percent parasitemia, or the percentage of total red blood cells that are infected by parasites.

Giemsa smears can also be used to identify life cycle stages (ring, trophozoite, schizont, see Figure 2.1), for use in generating synchronized cultures. Parasite culture at predominately the ring stage is synchronized to by treatment with 5% (w/v) sorbitol for 10 min. Pelleted red blood cells are resuspended in 5 volumes of 5% sorbitol for every 1 volume of the cell pellet and shaken vigorously. After leaving the mixture undisturbed at room temperature for 10 min, 5 mL of CM is added, and the culture is centrifuged again at 1500 rpm for 3 min. The supernatant is aspirated off; this completes the 1st wash. The pellet is resuspended in 10 ml fresh CM and transferred to a new sterile culture flask. The culture is allowed to grow for 4 hours and then the procedure is repeated (this is the 2nd wash); at hour 40 the procedure is repeated again to yield triple-synchronized culture. Before each sorbitol wash, the culture should be smeared and checked to ensure it is in the ring stage.
Figure 2.1. *P. falciparum* Life Cycle Stages As Seen on a Giemsa Smear. Parasites progress from ring (left) to trophozoite (second through fourth images) to schizont (fifth image) before merozoites rupture out of the red blood cell (right). Modified and reprinted with permission from Gligorijevic 2006a, Copyright 2006 American Chemical Society.

2.2.3 Synthesis of Alkynylated Azido Chloroquine (AAzCQ)

Scheme 2.1. Synthesis of AAzCQ.
**N-(7-Chloro-4-quinolyl)-1,4-diaminobutane (1)** – (Gorka *et al.* 2013a; Lekostaj *et al.* 2008).

4.6130 grams (23.3 mmol) of 4,7-dichloroquinoline was heated to 110°C in 13 mL (11.4 grams, 130 mmol) 1,4-diaminobutane for 6 hours under nitrogen. After cooling to room temperature, 60 mL of 1 N NaOH was added to the reaction mixture, which was then diluted with 400 mL of water. The mixture was then extracted with dichloromethane, and washed with brine and water. The organic layer dried over sodium sulfate and the solvent was evaporated under vacuum, yielding 5.3797 grams (21.6 mmol, 92% yield) of a white solid. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ = 1.19 (bs, 2H), 1.64 (m, 2H), 1.87 (m, 2H), 2.83 (t, 2H), 3.30 (q, 2H), 6.00 (bs, 1H), 6.38 (d, 1H), 7.33 (dd, 1H), 7.73 (d, 1H), 7.94 (d, 1H), 8.52 (d, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ = 26.26, 30.99, 41.70, 43.41, 99.02, 117.55, 121.62, 125.18, 128.92, 134.82, 149.42, 150.16, 152.28.

**N-(7-Chloro-4-quinolyl)-N’-(N”-t-Boc-aminoethyl)-1,4-diaminobutane (2)** - (Gorka *et al.* 2013a; Lekostaj *et al.* 2008).

0.9249 grams (3.68 mmol) of N-(7-Chloro-4-quinolyl)-1,4-diaminobutane was dissolved in anhydrous dichloromethane with 1.0 g (6.31 mmol) N-t-Boc glycinal and 1.4244 g (6.71 mmol) sodium triacetoxyborohydride, and the mixture was allowed to stir overnight. After quenching the reaction with water, the organic layer was extracted with saturated sodium bicarbonate, dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*, yielding a yellow oil. Flash chromatography using a solvent gradient was used to purify the product. The solvent system started with 5% methanol in dichloromethane. The concentration of methanol was gradually increased to 30%, then the last of the compounds were eluted with 100% methanol. Drying the third aliquot from the column yielded 1.0637 g (2.741 mmol, 74% yield) of N-(7-Chloro-4-quinolyl)-N’-(N”-t-Boc-aminoethyl)-1,4-diaminobutane. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ = 1.38 (s, 9H plus solvent peak overlap = 13H), 1.52 (m, 2H), 1.70 (m, 2H), 2.47 (m, 4H), 3.11
(m, 2H), 3.25 (m, 2H), 5.06 (bs, 1H), 5.63 (bs, 1H), 6.33 (d, 1H), 7.25 (dd, 1H), 7.79 (d, 1H), 7.78 (d, 1H), 8.42 (d, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta = 24.62, 25.98, 28.24, 38.33, 42.92, 49.90, 53.52, 79.06, 98.50, 117.01, 121.87, 124.90, 127.37, 134.78, 148.16, 150.32, 151.02, 156.19.

$N$-$(7$-Chloro-4-quinolyl)$-N'$-$(N''$-$t$-Boc-aminoethyl)$-N'$-$(3$-hydroxypropyl)$1,4$-diaminobutane (3) - (Gorka et al. 2013a; Lekostaj et al. 2008).

2.4355 grams (6.24 mmol) $N$-$(7$-chloro-4-quinolyl)$-N'$-$(N''$-$t$-Boc-aminoethyl)$-1,4$-diaminobutane and 0.5805 grams (0.30 mL, 3.12 mmol) 3-iodo-1-propanol were refluxed in anhydrous acetonitrile for 8 hours. The reaction mixture was concentrated in vacuo, yielding a brown-yellow oil. The oil was dissolved in ~60 mL of dichloromethane, extracted with saturated sodium bicarbonate, dried over anhydrous sodium sulfate, and concentrated in vacuo. The crude oil was purified by flash chromatography with 5% methanol in dichloromethane, followed by a gradual increase in the amount of methanol to 30%. Evaporation of the solvent, and drying under vacuum, yielded 1.2154 grams (2.69 mmol) of a brownish-yellow oil (86 % yield). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta = 1.37$ (s, 9H plus solvent peak overlap = 15H), 1.57 (m, 2H), 1.81 (m, 2H), 2.13 (m, 2H), 2.54 (m, 6H), 3.14 (m, 4H), 3.61 (m, 4H), 4.12 (bs, 1H), 5.14 (bs, 1H), 5.25 (bs, 1H), 6.69 (d, 1H), 7.51 (d, 1H), 7.82 (s, 1H), 7.94 (s, 1H), 8.62 (d, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta = 25.46, 25.74, 28.35, 30.91, 38.39, 43.35, 50.36, 51.82, 53.4, 57.48, 79.11, 98.30, 98.88, 116.72, 127.42, 127.84, 138.25, 140.73, 147.13, 155.02, 156.32.

$N$-$(7$-Chloro-4-quinolyl)$-N'$-$(N''$-aminoethyl)$-N'$-$(3$-hydroxypropyl)$1,4$-diaminobutane (4) - (Gorka et al. 2013a; Lekostaj et al. 2008).

0.1262 grams (0.28 mmol) $N$-$(7$-Chloro-4-quinolyl)$-N'$-$(N''$-$t$-Boc-aminoethyl)$-N'$-$(3$-hydroxypropyl)$1,4$-diaminobutane was dissolved in 10 mL anhydrous methanol. 2 mL of 2 M
HCl (4 mmol) was added, and the reaction was left to stir overnight. After concentration *in vacuo*, the product was dissolved in dichloromethane, and extracted with 1 M NaOH. The combined organic layers were dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*, yielding 0.077 grams (0.22 mmol, 78%) of a brown oil. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ = 1.53 (m, 2H), 1.70 (m, 2H), 1.85 (m, 2H), 2.42 (m, 4H), 2.66 (m, 4H), 3.22 (t, 2H), 3.52 (t, 2H), 3.91 (t, 2H), 6.29 (d, 1H), 7.24 (d, 1H), 7.74 (d, 1H), 7.83 (d, 1H), 8.40 (d, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ = 25.13, 30.60, 39.51, 48.55, 53.49, 54.26, 57.06, 57.62, 78.89, 98.60, 98.65, 114.16, 123.15, 127.03, 136.18, 139.03, 154.30, 156.05.

$N$-((7-Chloro-4-quinolyl)-$N'$-(2-aminoethyl)-$N'$-(3-hydroxypropyl)-$N''$-(4-pentynlamidyl)-1,4-diaminobutane (5) - Adapted from Hsu *et al.* 2007.

0.372 grams (1.06 mmol) $N$-(7-Chloro-4-quinolyl)-$N'$-(aminoethyl)-$N'$-(3-hydroxypropyl)1,4-diaminobutane dissolved in dimethylformamide along with 0.102 grams (1.04 mmol) 4-pentyneic acid, 0.826 grams (2.18 mmol) O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, and 0.1 mL (0.367 g, 2.85 mmol) diisopropylethylamine. The mixture was allowed to react at room temperature for 3 hours. The mixture was concentrated *in vacuo*, and the residue was dissolved in chloroform and extracted with saturated sodium bicarbonate. The organic phase was then washed with 1 M NaOH, dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*, yielding 0.379 grams (0.879 mmol, 84% yield). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ = 1.53 (m, 2H), 1.74 (t, 2H), 1.89 (s, 1H), 2.12 (t, 2H), 2.44 (m, 4H), 2.54 (m, 4H), 3.28 (m, 4H), 3.69 (t, 2H), 3.96 (t, 2H), 4.59 (t, 1H), 6.75 (d, 1H), 7.42 (m, 1H), 7.64 (m, 1H), 7.86 (m, 1H), 8.53 (d, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ = 15.61, 26.62, 28.75, 34.29, 35.92, 38.87, 44.68, 52.70, 54.94, 59.06, 62.79, 70.38, 83.57, 100.14, 112.38, 119.01, 126.68, 128.60, 140.15, 141.73, 148.44, 157.10, 174.52.
**Alkynylated Azido-Chloroquine (AAzCQ)** - Adapted from (Lekostaj et al. 2008).

0.321 grams (1.37 mmol) 8, 0.147 grams (1.21 mmol) 4-dimethylaminopyridine (DMAP), and 0.284 grams (1.48 mmol) EDC·HCl were placed under a nitrogen atmosphere. 0.189 grams (0.439 mmol) of N-(7-Chloro-4-quinolyl)-N’-(2-aminoethyl)-N’-(3-hydroxypropyl)-N”-(4-pentynlamidyl)-1,4-diaminobutane was dissolved in anhydrous dichloromethane, and injected into the flask, along with anhydrous dimethylformamide. The final solvent was 25% dimethylformamide in dichloromethane. The mixture was allowed to stir 4 days at room temperature. The reaction was dried and the residue was dissolved in dichloromethane, extracted with water, and washed with brine and sodium bicarbonate. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo*. Column chromatography was run using a gradient elution (5-50% methanol in dichloromethane), yielding a fraction that contains AAzCQ plus residual DMAP-8. A second column was used to purify AAzCQ, using a gradient elution of 10-50% methanol in ethyl acetate, followed by 100% methanol to ensure full elution. The column yielded 14 mg of AAzCQ (0.022 mmol, 5% yield). $^1$H-NMR (400 MHz, CD$_3$OD) $\delta = 1.25$ (m, 2H), 1.59 (m, 2H), 1.74 (m, 2H), 2.20 (s, 1H), 2.37 (m, 4H), 2.56 (m, 4H), 3.13 (m, 2H), 3.27 (m, 4H), 3.48 (m, 2H), 6.68 (d, 1H), 7.51 (dd, 1H), 7.78 (d, 1H), 8.20 (d, 1H), 8.32 (d, 1H). $^{13}$C-NMR (100 MHz, CD$_3$OD) $\delta = 15.70, 25.62, 26.95, 28.82, 36.03, 38.41, 39.42, 44.34, 54.53, 54.99, 70.31, 80.09, 83.53, 99.72, 117.90, 124.08, 125.13, 127.25, 138.55, 140.56, 142.94, 145.04, 145.44, 148.24, 155.09, 158.57, 165.01, 174.09. $^{19}$F-NMR (376 MHz, CD$_3$OD) $\delta = -154.66$ (m, 2F), -145.70 (m, 2F).

**Methyl Pentafluorobenzoate** (6) - Adapted from (Lekostaj et al. 2008).

5.0 grams (23.6 mmol) pentafluorobenzoic acid was dissolved in 30 mL of anhydrous methanol, and 1.6 mL of concentrated sulfuric acid was added. The mixture was refluxed for 24 hours. The
reaction mixture was concentrated \textit{in vacuo}, dissolved in dichloromethane, and extracted with saturated sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate, and concentrated \textit{in vacuo} to yield 3.5237 grams (15.6 mmol, 66% yield) of a yellow oil. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta = 3.96$ (s, 3H). $^{19}$F-NMR (376 MHz, CDCl$_3$) $\delta = -161.09$ (m, 2F), -149.15 (tt, 1F), -138.64 (m, 2F).

**Methyl 4-Azidotetrafluorobenzoate (7)** - Adapted from (Lekostaj et al. 2008).

3.5237 grams (15.6 mmol) of methyl pentafluorobenzoate was dissolved in 33.0 mL of a 8:3 acetone:water mixture. 1.0182 grams (15.7 mmol) of sodium azide was added, and the mixture was refluxed for 5 hours. The solvents were evaporated, yielding a crude light brown solid. The dried reaction mixture was dissolved in diethyl ether and extracted with water. The combined organic phases were dried over anhydrous sodium sulfate and the solvent removed \textit{in vacuo}, yielding 3.5123 grams (14.1 mmol, 90% yield) of white powder. $^1$H-NMR (400 MHz, CD$_3$OD) $\delta = 3.97$ (s, 3H). $^{19}$F-NMR (376 MHz, CD$_3$OD) $\delta = -153.78$ (m, 2F), -142.10 (m, 2F).

**4-Azidotetrafluorobenzoic Acid (8)** - Adapted from (Lekostaj et al. 2008).

0.4290 grams (1.716 mmol) methyl 4-azidotetrafluorobenzoate was dissolved in 10 mL of methanol and 1 mL of water. 1.1 mL (5.5 mmol) of 20\% NaOH was added, and the reaction was left to stir overnight. After acidifying the reaction solution to a pH<2 with 2 M HCl, the reaction mixture was extracted with ethyl acetate and dried over anhydrous sodium sulfate. The solvent was evaporated \textit{in vacuo}, yielding 0.3251 grams (1.381 mmol) of a light brown solid, an 80\% yield. $^1$H-NMR (400 MHz, CD$_3$OD) no resonances seen. $^{19}$F-NMR (376 MHz, CD$_3$OD) $\delta = -153.96$ (m, 2F), -142.25 (m, 2F).
2.2.4 Synthesis of Chloroquine Analogs

Compounds 1-4 of the AAzCQ synthesis were used in a study of chloroquine analogs, with the labels CQ-6, CQ-7, CQ-8, and CQ-9, respectively. This list was supplemented by a series of compounds synthesized previously (Natarajan et al. 2008; Yearick et al. 2008; Iwaniuk et al. 2009).

Figure 2.2. Structures of Quinolines and Chloroquine Analogs. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)
2.2.5 Synthesis of *epi*-Quinine and *epi*-Quinidine

Synthesis of *epi*-quinine and *epi*-quinidine was carried out by fellow Roepe group member Mr. Alexander Gorka (Gorka *et al.* 2013d).

![Scheme 2.2. Synthesis of *epi*-Quinine and *epi*-Quinidine by one-pot Mitsunobu esterification-saponification. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka *et al.* 2013d)](image)

**Synthesis of eQN and eQD**

(5)-(6-Methoxyquinolin-4-yl) [(2S,4S,8R)-8-vinylquinuclidin-2-yl]methanol (eQN) and (R)-(6-methoxyquinolin-4-yl) [(2R,4S,8R)-8-vinylquinuclidin-2-yl]methanol (eQD) were prepared by one-pot Mitsunobu esterification-saponification (Sidorowicz, *et al.* 2011). A stirred solution of QN or QD (500 mg, 1.5 mmol, 1.0 equivalent), triphenylphosphine (Ph$_3$P; 525 mg, 2.0 mmol, 1.3 equivalents), and *p*-nitrobenzoic acid (PNBA; 284 mg, 1.7 mmol, 1.1 equivalents) in anhydrous tetrahydrofuran (THF; 15 ml) was cooled to 0°C in an ice water bath, and diisopropyl azodicarboxylate (DIAD, 0.33 ml, 1.7 mmol, 1.1 equivalents) was added drop wise. The
resulting mixture was stirred at 0°C for 20 min, allowed to warm gradually to room temperature, and stirred for an additional 3 h. After cooling to 0°C, lithium hydroxide (LiOH; 1 M, 10 ml) and MeOH (2 ml) were added, the solution was gradually warmed to room temperature again, and the reaction mixture was stirred for 12 h more. Organic solvents were removed in vacuo, and the residue was quenched with water (10 ml) and extracted with dichloromethane (CH₂Cl₂; 50 ml). The organic phase was separated, washed with saturated brine, and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to afford the crude product as a yellow oil. This was further purified by flash chromatography over silica using an initial CHCl₃-diethyl ether (3:1) mobile phase followed by CHCl₃-MeOH-triethylamine (40:1:4) for product elution. The pure product was obtained as an off-white solid in 28% (eQN) or 31% (eQD) yield.

eQN.

¹H NMR (CDCl₃; 400 MHz; m, multiplet; s, singlet; d, doublet; dd, doublet of a doublet) δ 1.00 (m, 1H), 1.26 (m, 1H), 1.51 (m, 1H), 1.70 (m, 3H), 2.40 (s, 1H), 2.89 (m, 2H), 3.38 (m, 3H), 3.95 (s, 3H), 5.00 (m, 2H), 5.16 (d, J = 10 Hz, 1H), 5.72 (m, 1H), 7.37 (dd, J = 2.8 Hz, J = 9.2 Hz, 1H), 7.40 (d, J = 4.4 Hz, 1H), 7.68 (d, J = 2.8 Hz, 1H), 8.02 (d, J = 9.2 Hz, 1H), 8.72 (d, J = 4.4 Hz, 1H); ¹³C NMR (CDCl₃, 100 MHz) δ 24.7, 27.0, 27.1, 29.6, 39.1, 40.9, 55.3, 55.7, 61.6, 70.7, 102.5, 115.3, 120.1, 121.5, 128.0, 131.6, 140.3, 143.9, 144.8, 147.5, 157.6; melting point, 183 to 185°C; [α]²²D (EtOH) +23 (c 1.0); MS (ESI) m/z calculated for C₂₀H₂₄N₂O₂: 324.18. Found (M + H)⁺: 325.1.

eQD.

¹H NMR (CDCl₃, 400 MHz) δ 1.04 (m, 1H), 1.32 (m, 1H), 1.60 (m, 2H), 1.72 (s, 1H), 2.35 (m, 1H), 3.02 (m, 6H), 3.93 (s, 3H), 5.13 (m, 3H), 5.89 (m, 1H), 7.36 (dd, J = 2.8 Hz, J = 9.2 Hz, 1H), 7.48 (d, J = 4.4 Hz, 1H), 7.58 (d, J = 2.4 Hz, 1H), 8.02 (d, J = 9.2 Hz, 1H), 8.74 (d, J = 4.4 Hz, 1H).
Hz, 1H); $^{13}$C NMR (CDCl$_3$, 100 MHz) δ 23.9, 26.4, 27.3, 38.7, 46.8, 49.1, 55.4, 62.4, 69.8, 101.9, 115.0, 120.0, 121.7, 128.0, 131.6, 139.8, 144.7, 144.8, 147.5, 157.5; melting point, 185 to 187°C; [α]$^{22}_D$ (EtOH) +75 (c 1.0); MS ESI m/z calculated for C$_{20}$H$_{24}$N$_2$O$_2$: 324.18. Found (M + H)$^+$: 325.0.

2.2.6 β-Hematin Inhibition Assay

The initial design of this assay was completed prior to beginning research in the lab:


2.2.6.1 Effect of SDS on absorbance of FPIX (Gorka et al. 2013a).

The effect of SDS on FPIX absorbance was analyzed in two ways. First, a solution of FPIX (5 mM) was prepared in 0.1 M NaOH and diluted to 10 μM in 0.1 M bicarbonate buffer (pH 9.1), and aliquots of an SDS stock solution (50%, wt/vol; 1.734 M) were added to 1 ml of the FPIX solution in a stirred cuvette. Titration with SDS (to a final concentration of 0.5 to 5%, wt/vol) was monitored with an Agilent 8453 UV-visible spectrophotometer (Santa Clara, CA). Also, a 5 mM FPIX stock was diluted to 1 mM in 0.1 M bicarbonate buffer (pH 9.1) containing different concentrations of SDS (0, 0.5, 1.0, 2.5, and 5%, wt/vol). These solutions were then serially diluted in 1.5-μl microcentrifuge tubes such that the final FPIX concentration ranged from 0 to 20 μM. Equal volumes (200 μL) of these solutions were then transferred in triplicate to
microtiter plate wells. Absorbance readings from the wells were obtained at 405 nm using a BioTek ELx800 absorbance microplate reader (Winooski, VT).

2.2.6.2 Assay optimization (Gorka et al. 2013a).

The heme concentration was optimized by comparing the Hz yield upon various starting heme concentrations. The optimal concentration of catalyst was determined in the presence of 100 μM FPIX, propionate buffer (1 M) at 37°C in a 16-h assay format. We compared the Hz yield in the presence of sonicated lipids, phosphatidylserine (PS), PC, pMAG, sMAG, DO, DG, and DT, as well as mixtures of PC, pMAG, and sMAG, versus pH and at a range of lipid concentrations. PC, pMAG, and sMAG produced the highest Hz yields when used at ≥0.5 mg/ml. Additionally, catalytic efficiency was assessed at pH 5.2 versus pH 5.6 (Huy et al. 2007) in kinetic assays using PC, pMAG, or sMAG. The optimal buffer concentration was determined in the presence of 100 μM FPIX, propionate buffer (pH 5.2), and 0.5 mg/ml PC at 37°C using a 16-h assay as described below, with the greatest Hz yield achieved at 1 M propionate.

2.2.6.3. Hz inhibition assay (Gorka et al. 2013a).

The optimized assay is based on the differential solubility of crystalline and noncrystalline forms of FPIX in 2.5% (wt/vol) SDS (86.7 mM) and alkaline bicarbonate buffer (0.1 M, pH 9.1). Hemin (2 mM) in 0.1 M NaOH was titrated to the desired pH with propionate buffer. Ten-microliter volumes from the hemin stock were then transferred to 96-well plates, followed by addition of propionate buffer (180 μL per well) and previously sonicated lipid catalyst (10 μL). Appropriate blanks and controls (e.g., no lipid catalyst, no drugs) were included in each plate. Plates were wrapped in plastic wrap and incubated at 37°C with gentle shaking. Hz
yield assays were terminated after 16 h by addition of 100 μL of a solution of SDS dissolved in 0.1 M bicarbonate buffer (pH 9.1) (final concentration/well of SDS, 2.5%, wt/vol; 86.7 mM), and kinetic assays at multiple time points were quenched in a similar fashion. The well contents were gently mixed and incubated at room temperature for 10 min to allow undissolved crystals to settle. A 50-μL aliquot from each well was then transferred to a second plate preloaded with 200 μL/well of SDS solution (2.5%, wt/vol; 86.7 mM) in 0.1 M bicarbonate buffer, and the absorbance at 405 nm was read with a 96-well-plate-adapted ELx800 BioTek absorbance microplate reader.

Conversion of the absorbance values to the FPIX concentration (μM) remaining in the sample wells was done using a linear calibration curve measured for each assay. FPIX stocks ranging in concentration from 0 to 16 μM were freshly prepared in 2.5% SDS–0.1 M bicarbonate buffer (pH 9.1). Volumes of 250 μL were transferred to predesignated wells, the absorbance at 405 nm was read, and the data were fit to a straight line. Free FPIX remaining in solution (inverse of Hz produced) was quantified using equation 1:

\[
[H^x] = \frac{(A_{405}^s - A_{405}^0) - C}{\varepsilon_{405}} \times D
\]

where \([H^x]\) is the concentration of FPIX (μM) remaining, \(A_{405}^s\) and \(A_{405}^0\) are the absorbance (405 nm) readings (averages from triplicate wells) of the sample and blank, respectively, \(\varepsilon_{405}\) is the extinction coefficient for FPIX, \(C\) is a constant obtained from the fit to the calibration curve, and \(D\) is the dilution factor.

2.2.6.4. Vacuolar Accumulation Ratio Calculations (Gorka et al. 2013a).

Vacuolar accumulation ratio (VAR) calculations were done using the Henderson-Hasselbalch equation (Hawley et al. 1996; Krogstad et al. 1998). (see equation 2 below) with the external pH...
at 7.33 and the DV pH at either 5.2 or 5.6 (Bennet et al 2004b). Our calculations also assume that (i) charged (protonated) drugs are $>10^5$-fold less membrane permeant than neutral drugs and (ii) initial rapid accumulation is not limited by binding to the drug target(s):

\[
\frac{[\text{Drug}]_V}{[\text{Drug}]_E} = \frac{1 + 10^{(pK_a_1 - pH_v)} + 10^{(pK_a_1 + pK_a_2 - 2 pH_v)} + 10^{(pK_a_1 + pK_a_2 + pK_a_3 - 3 pH_v)}}{1 + 10^{(pK_a_1 - pH_E)} + 10^{(pK_a_1 + pK_a_2 - 2 pH_E)} + 10^{(pK_a_1 + pK_a_2 + pK_a_3 - 3 pH_E)}}
\]

where $V$ denotes DV, $E$ denotes external (blood), and $pK_a_1$, $pK_a_2$, and $pK_a_3$ correspond to titratable N on the CQ analogues. DV-scaled in vivo activities for drugs were obtained by multiplying the raw antiplasmodial IC$_{50}$s and LD$_{50}$s by the corresponding calculated VAR values.

### 2.2.7 Drug-Heme Affinity Measurements (Gorka et al. 2013d).


Drug-heme affinity measurements, made primarily by Roepe group member Dr. Alexander Gorka, were performed by monitoring the changes in the absorbance of the Soret band of heme in the presence of increasing concentrations of drug. Hemin was dissolved in DMSO or 0.1 M NaOH to 5 mM, followed by serial dilution to 5 μM in 40% DMSO–0.2 M HEPES (pH 7.4) for measuring binding to monomer (Casabianca et al. 2008). Drug solutions
were prepared by dissolving the compound in DMSO and diluting to 3 mM in 40% DMSO–0.02 M HEPES (pH 7.4). A cuvette containing 3 ml of freshly prepared heme (5 μM) was titrated with increasing concentrations of drug (final concentration, 0 to 210 μM), the sample was mixed following each addition, and the absorbance of heme at 402 nm was recorded using an Agilent 8453 UV-visible (VIS) spectrophotometer (Santa Clara, CA). Solvent dilution controls were similarly performed (final volume dilution, 6.54%) using the relevant drug-free medium.

Spectral and data analyses were performed using Kaleida Graph and SigmaPlot (version 11.0) software. Nonlinear least-squares curve fitting of the raw data was done using the Levenberg-Marquardt algorithm (Egan et al. 1997; Marques et al. 1996) (initial affinity coefficient [K_a] input, 0.01 μM\(^{-1}\)) and K_a's were computed.

2.2.8 Heme aggregation studies (Gorka et al. 2013d).


I measured the effect of various drugs on the pH-dependent solubility of FPIX as previously performed (Ursos et al. 2001), with minor modification. Briefly, drug stocks were prepared in 24 mM morpholineethanesulfonic acid (MES)-Tris buffer–0.1 M NaCl (pH 6.6) to a concentration of 1 mM. Hemin was dissolved in 0.1 M NaOH to 18 mM. Test solutions were
prepared by combining 20 μL of drug, 10 μL of FPIX, and 980 μL of 24 mM MES-Tris buffer–0.1 M NaCl at the desired pH, for final drug and FPIX concentrations of 20 μM and 178 μM, respectively. Samples were mixed and incubated at room temperature for 30 min, during which time aggregates of FPIX form that are visible to the naked eye. These aggregated FPIX species were pelleted by centrifugation at $1.61 \times 10^4 \times g$ for 3 min using an Eppendorf 5415 D microcentrifuge (Hauppauge, NY), removing the aggregated FPIX from that which is still soluble at the pH tested. The supernatant, containing the soluble FPIX, was removed for quantitative analysis to determine concentration using UV-Vis spectroscopy, taking advantage of the 405 nm absorbance profile of FPIX, see Figure 3.5A for the UV-Vis absorbance spectrum of FPIX.

In order to ensure that the transmitted light exceeds the lower limit of detection, the supernatant is added to a 96-well plate containing 24 mM MES-Tris buffer–0.1 M NaCl (pH 6.6), resulting in a 1:4 (vol/vol) dilution. A standard curve of known FPIX concentration was generated for each assay. The absorbance at 405 nm was measured using a 96-well-plate-adapted ELx800 BioTek absorbance microplate reader (Winooski, VT). Data were analyzed using the Microsoft Excel 2007 program, calculating the initial concentration of FPIX that remained in solution before the 1:4 dilution. This concentration was plotted vs. the pH examined, and the pH at half FPIX solubility (pH$_{1/2}$) was extracted from sigmoidal curve fits to FPIX concentration-versus-pH data using SigmaPlot (version 11.0) software. Values are averages of three replicates, each performed in triplicate (nine determinations total), and are reported ± SEM.
2.2.9 Antiplasmodial Assays

Antiplasmodial cytostatic (growth inhibitory, or IC\text{50}) and cytocidal (cell killing, or LD\text{50}) activity was determined for the strains discussed above essentially as previously described (Bennet et al. 2004a; Paguio et al. 2011), with minor modifications. The cytocidal assay utilizes a 6 h bolus dose with high concentrations of drug followed by washing drug away and growth in the absence of drug for 48 h, while the cytostatic assay utilizes continuous growth for 72 h in the constant presence of low concentrations of drug. For both assays, test compounds were dissolved in deionized water, 50\% EtOH, or DMSO.

In the cytostatic assay, serial drug dilutions were made using complete media and 100 \mu L aliquots were transferred to 96-well clear-bottom black plates. Culture for plate examination is prepared by generating a Giemsa smear to count parasitemia (see Section 2.2.2). Enough culture is prepared to add 100 \mu L to each well that contains drug concentrations or the no-drug control. From that total volume, the total red blood cell pellet is set at 4\% (4\% hematocrit). From the calculated parasitemia, the culture parasitemia is set at 1\%, by “diluting” the pellet with fresh uninfected red blood cell using a standard \text{Concentration}_1 \times \text{Volume}_1 = \text{Concentration}_2 \times \text{Volume}_2 calculation. Following addition of 100 \mu L of asynchronous or sorbitol-synchronized culture to the plated drug concentrations, the cultures in the plate are now at 1\% parasitemia, 2\% hematocrit) (see Section 2.2.2 for a full description of synchronous culture), plates were transferred to an airtight chamber gassed with 5\% CO\text{2}/5\% O\text{2}/90\% N\text{2} and incubated at 37 °C.

For the cytocidal assay, drug/parasite mixture was incubated for 6 h followed by centrifugation with an Eppendorf 5415 D microcentrifuge (Hauppauge, NY) at 1800 rpm for 1 min. Drug-containing media was removed and cell pellets resuspended three times with drug-
free complete media, using the same centrifuge settings, before plating of washed samples into a 96-well plate.

Washed plates along with the cytostatic assay plates were incubated at 37 °C for 48 h. After 48 h, 50 μL of 50X SYBR Green I dye (diluted using complete media from a 10,000X DMSO stock) was added and plates incubated for an additional 1 hr at 37 °C to allow DNA intercalation. Fluorescence was measured at 538 nm emission (485 nm excitation) using a Spectra GeminiEM plate reader (Molecular Devices; Sunnyvale, CA) fitted with a 530 nm long-pass filter. Linear standard curves of measured fluorescence vs. known parasitemia were prepared immediately prior to plate analysis. Background controls included fluorescence from un-infected red blood cells. Data was analyzed using MS Excel 2007 and IC$_{50}$ and LD$_{50}$ values obtained from sigmoidal curve fits to % growth/survival vs. drug concentration data using SigmaPlot 11.0. Reported values are the average of three independent assays, with each assay conducted in triplicate (nine determinations total) and reported ± standard error of the mean (S.E.M.), unless otherwise noted.

2.2.10. Trypan Blue Staining of *P. falciparum*

Staining of live versus dead parasites was carried out using the dye Trypan Blue (0.4%) (Strober 1997), from MP Biomedical (Santa Ana, CA). Briefly, synchronized parasites in the trophozoite stage (see Section 2.2.2. for description of synchronous culture) were magnetically enriched using a MACS LD column (Miltenyi Biotec, Inc., Auburn, CA) that utilize superparamagnetic ferromagnetic beads and a strong magnet to isolate paramagnetic infected RBCs from unininfected RBCs. The columns, which had a void volume of 1.35 mL, were run as follows: 2 mL serum-free media (to condition the column and prepare it for culture), 2 mL
culture (initially 10 mL at 2% hematocrit, resuspended as 2 mL at 10% hematocrit), and 2 mL
serum-free media (to rinse the uninfected red blood cells off). 2 mL serum-free media forced
through, in the absence of the magnetic field to release the infected red blood cells. The enriched
sample was centrifuged and the serum-free media was aspirated. The remaining pellet (volume
varied due to differences in parasitemia) was suspended in 0.08% saponin for 5 minutes, to
permeabilize the red blood cell membrane. An equal volume of trypan blue was added, and the
sample was mounted for microscopic examination at 100x (final concentration of saponin was
0.04%) using a Micromaster Digital Microscope (Fisher Scientific) with Micron Software
(Westover Scientific). Images of 3 independent fields of view were taken, and the parasites were
counted stained versus unstained, at least 100 total. Negative control (no drug), and positive
control (24 h at 5xLD₅₀ CQ) were run with each sample (6 h at LD₅₀ CQ)

2.2.11. Drug Combination Assays Using the Chou-Talalay Method

The effect of combining two drugs together was assayed through use of the Chou-Talalay
method of fixed-ratio analysis (Chou et al. 1984; Suberu et al 2013). Briefly, the two compounds
were initially screened for their individual cytostatic and cytocidal activities, as described in
Section 2.2.9. Combination analysis was initially run at fixed ratios of these determined values
(1:1 unless noted). This results in a set of concentrations all at a multiple or fraction of the
compounds’ monotherapy IC₅₀s or LD₅₀s. A combination stock, set at four times the IC₅₀ or
LD₅₀, is serially diluted seven times to yield a range of concentrations that can generate a
combination growth or survival curve, see Table 2.1 for a diagram of the plate layout.
Table 2.1 . Diagram of Plate for Chou-Talalay Method Assay.

<table>
<thead>
<tr>
<th></th>
<th>HB3 (CQS)</th>
<th>Dd2 (CQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3</td>
<td>4  5  6</td>
</tr>
<tr>
<td>A</td>
<td>100 nM ATM/ 80 nM NVP</td>
<td>No drug HB3</td>
</tr>
<tr>
<td>B</td>
<td>50 nM ATM/ 40 nM NVP</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25 nM ATM/ 20 nM NVP</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>12.5 nM ATM/ 10 nM NVP</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>6.25 nM ATM/ 5 nM NVP</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3.13 nM ATM/ 2.5 nM NVP</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1.56 nM ATM/ 1.25 nM NVP</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.78 nM ATM/ 0.63 nM NVP</td>
<td></td>
</tr>
</tbody>
</table>

These samples are processed as described above, using conditions of either the cytostatic or cytocidal assay. The initial data analysis is the same, generating growth or survival data. These data are then plotted versus each drug’s individual concentrations. For each assay, there is a 50% point, termed the “pseudo”-IC\textsubscript{50} or “pseudo”-LD\textsubscript{50}, since it represents the activity versus one drug’s combination. This value is then used to generate a fractional inhibitory concentration (FIC) or fractional lethal dose (FLD) using the following equations for compounds A and B (Chou et al. 1984; Suberu et al 2013):

\[
FIC_A = \frac{Pseudo - IC_{50A}}{IC_{50A}}
\]

\[
FIC_B = \frac{Pseudo - IC_{50B}}{IC_{50B}}
\]
By summing these two values, a combination index (or FIC\textsubscript{index}, FLD\textsubscript{index}) can be generated (Chou et al. 1984; Suberu et al. 2013):

\[ (2) \quad FIC_{\text{Index}} = FIC_A + FIC_B \]

The FIC\textsubscript{index} of a combination can be used to assign synergy, additivity, or antagonism. The cut-off values for these designations vary (Bell 2005), but those selected for this work were (Matthews et al. 2013):

- Synergy - FIC\textsubscript{index} ≤ 1.0
- Additivity - 1.0 < FIC\textsubscript{index} ≤ 2.0
- Antagonism - FIC\textsubscript{index} > 2.0

FIC\textsubscript{index} and FLD\textsubscript{index} values were averaged from at least 2 independent trials (with standard error of the mean calculated).

### 2.2.12. High-Throughput Screening of Drug Combinations

Initial IC\textsubscript{50}-based screening for drug synergies were performed in the Thomas Laboratory, National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH).

*Parasites, parasite culture, quantitative high throughput drug assay and matrix combination screening*

Methods for the SYBR qHTS and calculation of IC\textsubscript{50} and definition of curve classes have been described (Yuan et al. 2009; Inglese et al. 2006; Dodge 2003). Plating of compounds in matrix formation using acoustic droplet ejection and numerical characterization of synergy, additivity and/or antagonism have also been described (Mathews Griner et al. 2014).
The compound screening collection consisted of most known antimalarials and an annotated set of all FDA approved drugs for all disease indications as well as commercially available molecules in advanced clinical stages of development. The latter were obtained by NCATS, from suppliers such as Tocris Bioscience, Selleck Chemicals, Santa Cruz Biotechnology and Sigma Aldrich (among others). Priority was given to compounds based on known or generally accepted mechanisms of action, clinical status, FDA approval, or novelty in MOA.

Quality Control Criteria

The quality control score is a numerical characterization of the quality of a combination that attempts to take into account single agent performance and the presence of noise in the dose combination region of the matrix (Mathews Griner et al. 2014).

Figure 2.3. Example of Heat Map Generated by High-Throughput Screening at NCATS.
It is composed of a number of heuristics, developed by examination of a series of matrix screening runs (Mathews Griner et al. 2014). The calculation, done at NCATS, is performed on the bounded (0-100) response matrix and considers the following conditions:

DMSO negative control response lies between 80 and 100. Both single agents should display a valid dose response curve and provide a valid IC\textsubscript{50} value. If single agents have a valid curve fit, the range of growth responses should have a relative standard deviation greater than 20 (Dodge 2003). For a combination matrix, the relative standard deviation of the dose combination sub-matrix should be greater than 25. The responses in the dose combination should also not exhibit random distribution across the matrix. This is measured by Morans I (Moran 1950). We consider a combination to exhibit spatial autocorrelation if the p-value is less than 0.05.

Each combination is tested against the five conditions and the result is expressed as a vector, defined as

\[ C = \{C_i\}, i = 1 \ldots 5 \]

where \( C_i = 1 \) if the i’th condition is true, 0 otherwise.

The final score is obtained by computing the dot product of the binary vector and a weight vector, \( W \) of the same length as \( C \). The latter allows us to assign an importance to each of the five conditions noted above. The final score is given by

\[
QCScore = \sum_{i=1}^{5} C_i W_i
\]

Currently, the weight vector is defined as \( W=\{2,5,3,5,3\} \), allowing for a minimum score of 0 (the combination passes all QC criterion) and maximum score of 18 (combination fails all QC criterion). A combination with a score of 0 is deemed as good quality and suitable for further consideration. Increasing values of the QCScore indicate poorer quality combination responses.
It should be noted that the score can identify false positive – combinations that are scored well, but on visual inspection turn out to be poor quality. The score is meant to provide an initial ranking and should not preclude manual inspection.

Figure 2.4. Example of an Unacceptable Heat Map Generated by High-Throughput Screen at NCATS.

In addition to the previously described metrics (Mathews Griner et al. 2014) analysis of combinations at NCATS to include two other quantifiers of synergy - DBsumPos and DBsumNeg. These are defined as the sum of positive deviations from the Bliss model and the sum of the negative deviations from the Bliss model, which defines synergy and antagonism in terms of predicted additive activity as the sum of the monotherapy activities minus their product. Positive deviations in the observed activity denote antagonism, while negative deviations denote synergy (Petratis et al. 2009). In contrast to simply summing all the deviations from the Bliss
model, these two variables characterize the extent of synergy and antagonism within a set of dose combinations.

2.2.13. Photolabeling PfCRT Proteoliposomes with AzBCQ and AAzCQ, (adapted from Lekostaj et al. 2008).

Proteoliposomes (PLs) containing purified PfCRT with hexa-histidine and V5 epitope tags were synthesized as described in Lekostaj et al. 2008 by Mr. Paul Callaghan of the Roepe group. 0.1 nmol PfCRT was suspended in a 50 mM MES/Tris buffer and mixed with diluted AzBCQ or AAzCQ, with or without the presence of underivatized (“cold”) CQ, to achieve the desired concentration in a 96-well plate. The plate was mixed at 650 rpm for 30 sec in the MixMate (Eppindorf) and incubated for 10 min at 37°C. The samples were then illuminated with 254 nM UV light using a hand-held lamp (Spectroline model ENF-280C, 115 V, 60 Hz, 200 mA; 8 W bulb emitting approximately 500 μW/cm² approximately 10 cm from the bulb surface) for 10 minutes. Samples were quenched by mixing with an equal volume of 2x click solution for a final solution containing 150 μM Biotin Azide, 200 μM copper(II) sulfate, 2 mM sodium ascorbate, 200 μM bathophenanthroline disulfonic acid, and 1% sodium dodecyl sulfate (SDS). AzBCQ samples were just diluted with SDS-containing water. The samples were incubated for 30 minutes while mixing at 500 rpm on the MixMate for 30 min. Protein was precipitated with 50% trichloroacetic acid (TCA) (one-third the sample volume), incubated at -80°C for 20 min, and pelleted at 13,200 rpm in an Eppendorf 5415 D microcentrifuge (Hauppauge, NY), followed by washing with 1:1 EtOH:diethyl ether three time. Samples were then prepared for SDS-PAGE separation by dissolving with Laemmlı buffer. Samples are run on a 12% acrylamide gel for 1
hour at 150 V, and transferred onto a PDVF membrane overnight at 50 mA for Western Blot analysis with streptavidin-HRP and either anti-hex-his or anti-V5 HRP.

2.2.14. Photolabeling *P. falciparum* with AzBCQ and AAzCQ for Fluorescence Microscopy.

Synchronized trophozoite cultures were photolabeled with AzBCQ and AAzCQ by UV-irradiation at 254 nM using a hand-held lamp (Spectroline model ENF-280C, 115 V, 60 Hz, 200 mA; 8 W bulb emitting approximately 500 μW/cm² approximately 10 cm from the bulb surface). Various incubation times of cells in probe-containing media were used before illumination, which was varied in its time. Following fixation of the sample with 4% formaldehyde and 0.0075% glutaraldehyde for 30 min at 37°C, membranes were permeabilized with 0.1% Triton-X at 37°C for 10 minutes (Tonkin et al. 2004; Gaviria et al. 2013).

Cells labeled with AzBCQ were then incubated with a streptavidin-linked Alexa Fluor 488 conjugate, while AAzCQ-treated cells were blocked with azide-labeled TAMRA in the absence of coupling agents, and then coupled to azide-labeled Alexa Fluor 488. Coupling was performed using 200 μM copper(II) sulfate and bathophenanthroline disulfonic acid (a hydrophilic chelator- Gupta et al. 2005), with 800 μM sodium ascorbate to reduce copper(II) to copper(I). Cells were then adhered to poly-lysine-coated #1.5 coverslips and mounted using Fluorogel Mounting Media to glass slides for imaging on Nikon Eclipse TE 2000-U spinning disk confocal microscope with 405, 491, 561, and 642 nm laser lines at 200 ms exposure and 35% laser power. Image files, obtained using Differential Interference Contrast and Fluorescence Channels, were deconvolved using a point spread function with multiple iterations using AutoQuantX2. Images were processed and overlays constructed using and Imaris 7.4.2 Software.
as described in earlier Roepe group publications (see Gligorijevic et al. 2006a; Gligorijevic et al. 2008; Gaviria et al. 2013).

### 2.2.15. Photolabeling P. falciparum for Gel Electrophoresis and Proteomic Analysis

10% parasitemia cultures at 600 uL pellet volume in 30 mL of complete media were used for photolabeling. The pellet was washed 3 times with PBS and resuspended in 0.5% saponin (w/v) in PBS for 5 min at 37°C with occasional agitation. The isolated parasites were collected by centrifugation at 1500 g for 10 min, and washed 3 times with PBS. The parasites were resuspended in either 10 µM AzBCQ (2D gel electrophoresis) or 200 nM (HB3) or 15 µM (Dd2) AzBCQ or AAzCQ (avidin-enrichment) in PBS in a 96-well plate. The samples were incubated for 10 min at 37°C in the dark, and then illuminated with 254 nM UV light using a hand-held lamp (Spectroline model ENF-280C, 115 V, 60 Hz, 200 mA; 8 W bulb emitting approximately 500 µW/cm² approximately 10 cm from the bulb surface) for 10 minutes. The pellet was then washed 3 times with PBS, half was used for visualization with a biotin Western blot, and the other half for downstream mass spectroscopy through Coomassie Staining.

#### Two-Dimensional Gel Electrophoresis

For the 2D gel electrophoresis experiment, the second fraction was stored in PBS-Tween20. Stored samples were spun down and washed with PBS before precipitated with 50% trichloroacetic acid (one-third the sample volume) and washed three times with 1:1 EtOH:diethyl ether. Pelleted samples were resuspended in BioRad 2D Rehydration buffer (125 µL) (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte, 0.001% Bromophenol Blue) and incubated overnight to rehydrate pH 3-10 IPG strips. Strips were focused at 250 V for 20 min (linear increase), followed by 4,000 V for 2 hours (linear increase), then 4,000 V at 10,000 Vhrs
(rapid increase). The strips were then washed with BioRad Equilibration Buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% (w/v) DTT), Equilibration Buffer II (M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2.5% iodoacetamide), and running buffer (Tris-HCl/Glycine/SDS) before imbedding the strip into a pre-cast gradient gel (BioRad) and electrophoresed at 200 V for 36 min. Samples for biotin Western blot were transferred to a PVDF membrane overnight at 50 mA. Samples for mass spectroscopy proteomics were washed twice with deionized water, stained with Bio-Safe Coomassie stain (Bio Rad) overnight, washed twice with deionized water, and destained with 10% methanol/7.5% acetic acid in deionized water until the background was no longer stained.

Avidin-Affinity Enrichment and One Dimensional Electrophoresis

Following the probe crosslinking and the PBS wash, samples were mixed with and equal volume of a 2x click solution containing 150 uM azido biotin, 100 uM copper(II) sulfate and bathophenanthroline disulfonic acid, 1 mM sodium ascorbate and 2% SDS, if the sample was labeled with AAZCQ. AzBCQ samples were just mixed with an equal volume of 2% SDS. The proteins were precipitated with 50% TCA, and washed three times with 1:1 ethanol:diethyl ether before drying. Dried protein was resuspended in 1% SDS before loading onto 100 µL Pierce Avidin Agarose (Thermo Scientific, Waltham, MA) that had been washed three times with 1% SDS. The protein was incubated at room temperature on the avidin agarose with rotation for 1 hour. The agarose was then washed three times, saving the washes for running on a 1D SDS-PAGE gel after precipitation with TCA and washing with ethanol/diethyl ether. After the washes, proteins were eluted off the agarose by boiling for 2 min in 1x Laemmli buffer. Initial supernatant was run alongside each gel, with the first and third wash of each sample and the eluents. Half of the sample was loaded onto a gel for visualization with a biotin Western blot,
while the other half was loaded onto a companion gel for Coomassie Blue staining (see Two Dimensional Gel Electrophoresis) for mass spectroscopic proteomic analysis.

2.2.16. Mass Spectroscopy-Based Proteomics

Protein spots were excised from the gel in the Radoslav Goldman Laboratory (GUMC) and destained by 50% ACN in 10 mM ammonium bicarbonate. In-gel trypsin digestion was performed by adding 10 mM ammonium bicarbonate at pH 7.8 and 0.2 ug trypsin (Promega) followed by incubation at 37°C overnight. Peptides were extracted in 3 steps with gradient ACN 20-60% in 1% trifluoroacetic acid by sonication. Peptide mixture was separated by reversed phase chromatography (Tempo Eksigent-AB Sciex, Framingham, MA) on a ChromXP C18-CL (3μm, 120Å, 180μm, 20 mm) trap column and ChromXP C18-CL (3μm, 120 Å, 75μm, 150 mm) HPLC capillary chip column (Eksigent-AB Sciex) interfaced with a 5600 TripleTOF mass spectrometer (AB Sciex, Framingham, MA). Chromatographic method was composed from 10-min trapping/washing step (2% ACN, 0.1% formic acid (FA)) at 3 μL/min flow rate and 45 min analytical step/gradient elution at a flow rate of 300 nL/min (Solvent A: 2% ACN with 0.1% FA; Solvent B: 100% ACN with 0.1% FA) using timetable: 5-40% Solvent B 0-23 min; 45-100% Solvent B 23-25 min; 100% Solvent B 25-30min.

Mass spectrometric parameters were set to ion spray voltage 2400 V, ion source gas (GS1) 13, declustering potential 90 and interface heater temperature 150°C. Mass spectrometer was operated in data-dependent mode; 1x survey scan (m/z 400 to 1800) followed by the MS/MS experiments from 50 precursor ions selected for collision-induced dissociation, collision energy was set automatically according to charge state and m/z of precursor ion. Range for recording of MS/MS spectra was set to m/z 100 - 1600, with the dynamic exclusion time set to 6 sec, and 150
counts threshold, for two repeated precursor. Mass spectrometric data were processed by Protein pilot (AB Sciex) and Mascot (Matrix Science) software.

2.2.17. Immunofluorescence Assays of *P. falciparum* ATG8 Puncta

For drug treatments, highly synchronized mid stage trophozoites were treated as described (Paguio *et al.*, 2011) using drug concentrations noted in the text. Resultant cell pellets were resuspended in PBS and treated as below. Cells were washed 3 times with PBS, fixed with 4% formaldehyde/0.0075% glutaraldehyde in PBS for 30 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, reduced with 0.3 mg mL\(^{-1}\) sodium triacetoxyborohydride for 10 minutes, blocked with 5% goat serum for 1 hour, and sequentially treated with antibodies diluted in 5% goat serum/PBS Tween-20 with PBS Tween-20 washes in between. The primary antibody was raised in rabbit (TgATG8); the secondary antibody was raised in goat against rabbit and conjugated to DyLight 649 fluorophores (Jackson Immunoresearch). Cells were attached to #1.5 coverslips and mounted using “Fluorogel” mounting media. Samples were imaged using a customized Nikon Eclipse TE 2000-U spinning disk confocal microscope with 405, 491, 561, and 642 nm laser lines at 200 ms exposure and 35% laser power. For primary antibodies raised in rabbit, primary solutions were prepared at 1:250 and secondary solutions (goat anti rabbit DyLight649) at 1:500.

Images were iteratively deconvolved using a point spread function obtained under identical imaging conditions (via doping one sample with fluorescent beads) and running multiple iterations in AutoQuantX2 (Gligorejivic *et al.*, 2006). Images were further processed and overlayed using Imaris 7.5.2 software. Using the “spots” routine in Imaris 7.5.2, puncta were defined and distances were measured from each spot to a single point within the DV as
defined by the center of hemozoin optical density (Gligorejivc et al., 2006 and Gaviria et al., 2013). These distances were exported to excel and the resulting data were plotted as number of puncta vs. distance from hemozoin.

2.2.18. Single-Cell Photometry of *P. falciparum* Intracellular Ca\(^{2+}\) Concentrations.

For calibrations, perfusates were prepared from 2 stocks: 1) HBSS + 25 mM HEPES + 10 mM EGTA (pH 7.4); 2) HBSS + 10 mM EGTA + 25 mM HEPES + 10 mM CaCl\(_2\) (pH 7.4). Solutions of designated concentrations of free calcium were prepared by mixing both stocks at different ratios. The free calcium concentration was calculated using the “MAXCHELATOR” program (provided by Professor Chris Patton from Stanford University http://maxchelator.stanford.edu/index.html); parameters were set at pH 7.4, 37°C, EGTA, ionic strength 150 mM. Before experiments, 10 µM ionomycin was added in order to clamp the internal free calcium concentration to the concentration in the perfusate. Intact trophozoite-stage parasites were first perfused with calcium-free buffer (without ionomycin) at a rate of 1 mL/min. Ratiometric measurements were performed in 5 cells corresponding to resting free calcium. This process was repeated for each perfusate containing ionomycin and a designated level of calcium (54, 127, 217, 488, 1,000, and 23,700 nM); ratiometric data were collected for ~15 cells at each calcium concentration. Data were processed and analyzed with Microsoft Excel and SigmaPlot 11.0. Calibration curves were fitted to sigmoidal curves and calcium concentrations were extrapolated from the best fit.

Coverslips were prepared by pipetting 500 µL of 0.1% (w/v) poly-L-lysine over them and letting it sit for 10 min; they were then dried and stored at 4°C until used. Trophozoite-infected RBCs were resuspended at 0.5% hematocrit in IM with 25 mM HEPES pH 7.4 and 200 µL were
placed on a poly-L-lysine coated coverslip; the cells were incubated for 3 min under standard cell culture atmosphere. Non-adherent cells were washed off and the coverslip was mounted on a custom-designed perfusion chamber for microscopy work. For perfusion experiments, the parasites were always maintained under constant perfusion at 1 mL/min of a physiologic buffer (HBSS with 5% O₂, 5% CO₂, and 90% N₂ at 37º C). Fura-2 AM (in DMSO stocks) was added to the samples at a final concentration of 5 µM. 0.1% v/v Pluronic F-127 was added to improve dye loading into iRBCs. Samples were kept at 37 º C for 45 min. The ratiometric values of ~15 intact cells were measured for controls and each drug concentration.

The single cell photometry (SCP) apparatus consisted of a custom-built Nikon Diaphot epifluorescence microscope equipped with a 100X oil immersion objective capable of UV transmission (Fluoro, N.A. 1.25, 160/017); a 16-bit Sensys CCD camera (Tucson, AR) was attached to the side port of the microscope. Excitation light was provided by a computer controlled xenon arc lamp (LAMBDA LS, Novato, CA). 2 band-pass filters at 340 nm and 380 nm filtered UV light for ratiometric illumination of Fura-2 (Asahi Spectra Co., Ltd., San Jose, CA). The filters were housed in a Lambda-10 filter wheel controlled through the acquisition software (Imaging Workbench). The excitation light was transported by a liquid light guide (Novato, CA), collimated, and passed through the microscope optics. A filter cube housing a 400 nm dichroic mirror combined with a 410 nm long-pass filter was used to separate excitation from emission light. The power before objective was measured with a near UV power meter (Metrologic Model No. 45-545). In our experiments, the exposure time was set at 500 ms followed by a 10 sec recovery in total darkness. For ratiometric measurements, data from 2 excitations (340 and 380 nm) were collected from a region of interest (ROI) within the parasite. A second region of interest was drawn in the vicinity of the parasite for background subtraction.
To obtain the transient changes in cytosolic Ca\textsuperscript{2+} with cytostatic and cytocidal dosages of drugs, in real time, the drugs were perfused at the required concentration using a custom-designed perfusion chamber at 1 mL/min and the ratiometric measurements were obtained.
CHAPTER III

EXAMINING THE CYTOCIDAL VERSUS CYTOSTATIC EFFECTS OF ANTIMALARIAL DRUGS THROUGH BIOCHEMICAL GROWTH ASSAYS AND IN VITRO CHEMICAL METHODS

3.1 Background

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3.1.1. Part 1: The Need for Cytocidal Assays for *P. falciparum*

Recent work has shed light on the difference between growth-inhibitory (or cytostatic) effects and cell-kill (cytocidal) effects for antimalarial drugs. The commonly-referenced cause of CQR, that reduced accumulation in the DV limits its ability to inhibit the crystallization of heme, does not necessarily correlate with additional observations of reduced daughter nuclei and
reduced viability of the next generation following invasion following a cytocidal dose of CQ (Gligorijevic et al. 2008). Similarly, CQR parasites are able to survive intra-DV concentrations of CQ that are lethal to CQS stains (Cabrera et al. 2009a). Hz crystallization may be a target of low-dose growth inhibition, but the targets and resistance trends of cytocidal treatment still need to be defined.

The quantification of the cytocidal effects of antimalarial compounds on Plasmodia has not been perfected, despite the previous optimization of several different assays to quantify the cytostatic growth inhibitory effect (Bennet et al. 2004b). Assays that have been published include those that rely on the growth of limiting dilutions of culture to yield single parasite aliquots that will either grow or not grow in a binary measure of viability (Sanz et al. 2012), and an assay that relies on the overexpression of luciferase in transfectant parasites and the bioluminescence that the luciferase generates (Hasenkamp et al. 2013). In order to properly quantify death, one can in theory utilize cell viability assays such as the trypan blue exclusion assay. These assays rely on the ability of viable cells’ membranes to exclude certain dyes. When a culture of cells is mixed with one of these dyes, microscopy can be used to count those which exclude the dye, and are still viable, and those which are stained, which are dead (Hathaway et al. 1964; Strober 1997).

The use of live-dead cells stains such as trypan blue, however, are made more difficult due to the parasite’s location within the red blood cell and the parasitophorous vacuolar membrane. Those dyes that rely on membrane integrity to delineate viable cells would have to circumvent these other membranes before any degradation of the parasite plasma membrane can be observed. It is not known if the loss of viability in the parasite coincides with any alteration of the membrane integrity of the RBC membrane. However, infected RBCs are noted to have
increased permeability to select metabolites such as sodium and calcium ions, but the integrity is maintained to avoid osmotic swelling by the consumption of host Hb (Lew et al. 2003). Fully permeabilizing the RBC plasma membrane and the parasitophorous vacuole can be achieved through treatment with saponin, potentially allowing for the use of membrane-integrity dyes. The plant-derived detergent permeablizes cholesterol-containing membranes to macromolecules, while the parasites retain viability as demonstrated by: the ability to extrude trypan blue, metabolic inclusion of radio-labeled isoleucine, and the presence of millimolar levels of ATP within the parasite (Saliba et al. 1999). It may be possible to define cytocidal action through permeabilization of the outer membranes and staining with a dye like trypan blue. In part 1 of this chapter, I explore this idea.

3.1.2. Part 2: Is Hz a Cytocidal Target?

The Hz crystallization pathway is an important target for antimalarial chemotherapy at cytostatic doses, both whether it is relevant for cytocidal doses is not entirely clear. Quinoline antimalarials, such as CQ, quinine (QN) and amodiaquine (AQ), are known to bind to multiple precrystalline forms of heme in different ways and thus presumably inhibit crystal growth by sequestration of monomeric and/or dimeric heme (de Dios et al. 2004; de Dios et al. 2003; Egan et al. 2005; Egan et al. 1994; Leed et al. 2002; Sullivan et al. 1996a, reviewed in Gorka et al. 2013c). During the trophozoite stage of the intraerythrocytic cycle, P. falciparum actively degrades host red blood cell hemoglobin, producing millimolar quantities of toxic ferriprotoporphyrin IX (FPIX) heme within the digestive vacuole (DV) of the parasite (Banerjee et al. 2002; Gamboa de Domínguez et al. 1996). Due to the lack of a heme oxygenase pathway (Yoshida et al. 2000), the malaria parasite maintains a low concentration of toxic free heme by
sequestration into inert, nontoxic crystalline Hz (Egan et al. 2002; Pagola et al. 2000; Pisciotta et al. 2007; Tripathi et al. 2002).

Quantifying inhibition of Hz formation in vitro has often been viewed as a faster and more economical way to predict and even quantify antiplasmodial activity in vivo for some drugs (Egan et al. 2005; Egan et al. 1994; Dorn et al. 1998; Egan et al. 2001; Hawley et al. 1998; Huy et al. 2007; Kurosawa et al. 2000; Natarajan et al. 2008; Ncokazi et al. 2005; Parapini et al. 2000). However, the assays that have been used in this manner are typically done under conditions that are far from physiological. Furthermore, to our knowledge, no studies have tested how Hz inhibition potency is related to both IC$_{50}$ and LD$_{50}$.

Although Hz crystallization chemistry is not completely elucidated, it is known that preformed Hz, proteins, and lipids can facilitate heme crystallization (Pisciotta et al. 2007; Chong et al. 2003; Jackson et al. 2004; Sullivan et al. 1996b). Current evidence strongly supports lipid catalysis of crystallization in vivo, since lipid nanospheres have been shown to be closely associated with nascent Hz crystals within the DV (Pisciotta et al. 2007; Jackson et al. 2004). A variety of lipids isolated from the DV of malarial parasites have been shown to efficiently catalyze Hz crystal growth in vitro (Pisciotta et al. 2007; Jackson et al. 2004; Egan et al. 2006; Fitch et al. 1999). Although the concentration and exact identity of the lipids extracted from the DV is still not fully known (Pisciotta et al. 2007; Jackson et al. 2004), phosphatidylcholine (PC), monoacylglycerols (MAGs), diacylglycerols (DAGs), and triacylglycerols (TAGs) are among those identified to be present within this acidic compartment.

Several plate-based and non-plate-based assays have been developed to quantify Hz crystal growth and inhibition of that growth by drugs (Tripathi et al. 2002; Huy et al. 2007; Kurosawa et al. 2000; Ncokazi et al. 2005; Huy et al. 2006; Pandey et al. 1999a; Pandey et al.
Most previously reported assays require centrifugation and/or filtration and are performed under nonphysiological conditions (an exception being low-throughput assays performed previously [Pisciotta et al. 2007]). Some of these studies have hinted at a correlation between Hz inhibition potency and antiplasmodial IC₅₀, but contrasting results have also been reported (Dorn et al. 1999; Hawley et al. 1998; Kaschula et al. 2002). For previously reported high-throughput assays, multiple steps involved in the processing of Hz crystals are cumbersome and can add additional error. Some use hazardous reagents or require expensive equipment, such as microfiltration plates.

Additional development of several classes of antimalarial drugs would benefit from a more complete understanding of the relationship between drug potency against malarial parasites and their ability to inhibit Hz formation. As part of this effort, high-throughput assays that more closely mimic the physiological conditions of Hz formation would be helpful. The Roepe Lab has developed such an assay that uses physiologically relevant lipid catalysts, ionic strength, and temperature and that incorporates inexpensive, readily attainable, nonhazardous reagents (Gorka et al. 2013a). Using this assay and working alongside my colleague Dr. Alexander Gorka, I helped to quantify the effect of pH on Hz inhibition for common antimalarials, CQ, QN, AQ, and quinidine (QD) and evaluated the catalytic efficiencies of different lipids. We also tested for a correlation between Hz inhibition and antiplasmodial IC₅₀ and LD₅₀ for a series of CQ analogues with a wide range of antiplasmodial activities (Scheme 3.1) (Natarajan et al. 2008; Ekoue-Kovi et al. 2009; Iwaniuk et al. 2009; Yearick et al. 2008). The compounds I contributed, 6-9, allow for a range of side-chain properties including: primary, secondary, and tertiary amines that are either diprotic or triprotic, and with alcohol and carbamate functional groups.
3.1.3. Part 3: Cytocidality in QN vs. CQ

Rationally-designed improvement of quinoline-based antimalarials has historically focused on modification of the 4- and 8-aminoquinolines like CQ, whereas synthesis of quinine (QN) derivatives has largely been avoided due, in part, to toxicity concerns and complexities in chemical synthesis (Kumar et al. 2009). Yet QN, a quinoline methanol natural product from the bark of the *Cinchona* tree (Wells et al. 2009), has been used as an effective antimalarial drug for centuries, and QN resistance (QNR) remains relatively low. Correspondingly, QN is currently a WHO-recommended therapy for some chloroquine (CQ)-resistant (CQR) and artemisinin (ART)-resistant *P. falciparum* infections. Despite long-term use and important activity against drug-resistant malaria, the molecular mechanism of action of QN has not yet been fully

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**Scheme 3.1.** Structures of Common Antimalarials, and Chloroquine Analogs. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)
elucidated. Understanding similarities versus differences relative to CQ would assist with the additional development of effective quinoline antimalarial drugs.

Resistance to QN is multifactorial and in various studies has been linked to mutations and/or overexpression of several genes, including those encoding the *P. falciparum* chloroquine resistance transporter (PfCRT) (Cooper *et al.* 2002; Griffin *et al.* 2012), multidrug resistance protein (PfMDR1) (Sidhu *et al.* 2005), and Na⁺/H⁺ exchanger (PfNHE) (Ferdig *et al.* 2004). One theory for why QN remains active against some CQR phenotypes is that certain mutant PfCRT isoforms that confer reduced CQ accumulation via increased PfCRT-mediated transport of CQ do not accommodate increased transport of QN, even though CQ and QN are structurally similar (Cooper *et al.* 2007; Roepe 2011; Sidhu *et al.* 2002). Our laboratory has recently discovered that the mechanism of resistance to the cytoidal (cell-killing) activity of CQ is likely distinct from the mechanism of resistance to the cytostatic (growth-inhibitory) activity of CQ (Roepe 2011; Cabrera *et al.* 2009a; Paguio *et al.* 2011). Yet, other than one recent report (Paguio *et al.* 2011), there has been no quantification of the cytostatic versus the cytoidal doses for QN and its stereoisomers; therefore, it is currently not known how these parameters differ for various strains and isolates of *P. falciparum*.

The behavior of QN stereoisomers (see Scheme 3.2) is particularly interesting and is in theory a quite useful tool for elucidating QN pharmacology. Karle and coworkers reported that while QN (8S,9R) and its 8,9-diastereomer quinidine (QD) (8R,9S) potently inhibit the growth of malarial parasites, 9-epi-QN (eQN) (8S,9S) and 9-epi-QD (eQD) (8R,9R) do not, showing strongly elevated IC₅₀s against CQ-sensitive (CQS) strain D6 and CQR strain W2 (Karle *et al.* 1992). Curiously, W2 was found to be approximately 3-fold more sensitive to eQN and eQD than D6. Albeit under nonphysiological conditions, Egan *et al.* demonstrated that the 9-epimers
inhibit hemozoin formation less well in vitro than QN and QD (Egan et al. 1994). These findings, along with structural analysis of the compounds, led Karle et al. to hypothesize that the altered orientation of intermolecular hydrogen bonds with cellular receptor sites for the threo alkaloids eQN and eQD relative to that of their erythro counterparts, QN and QD (Karle et al. 1992), reduces Cinchona alkaloid potency. Recent work (Alumasa et al. 2011; Egan et al. 2005; Leed et al. 2002; Cooper et al. 2002; Sidhu et al. 2005; Ferdig et al. 2004; Cooper et al. 2007; Roepe 2011; Sidhu et al. 2002; Warhurst et al. 2003) has suggested that the cellular receptor sites alluded to by Karle et al. may be one or more forms of free FPIX heme within the DV.

Warhurst and coworkers investigated the physicochemical properties of the erythro versus threo isomers and calculated that the latter have an elevated aliphatic N pKa (9.5 versus 8.6 for the erythro isomer) and a reduced log D at pH 7.4, predicting decreased membrane transfer (Warhurst et al. 2003). Thus, intrinsic differences in the antiplasmodial activities of the isomer pairs may result from (i) a different chemical interaction(s) with heme, (ii) a decreased ability of eQN and eQD to partition from aqueous to lipid phase (i.e., to cross membranes) (Warhurst et al. 2003), or (iii) perhaps altered binding to other (nonheme) cellular receptors. In parasites that are resistant to quinoline drugs, mutations in DV membrane transporters with stereospecific drug interactions (Sidhu et al. 2005; Cooper et al. 2007; Roepe 2011; Sidhu et al. 2002) might be predicted to further affect 9-epimer activity for these QNR or CQR strains.

To further distinguish between these possibilities, we hypothesized, based on a recent model for QN-heme adducts (Alumasa et al. 2011), that it is possible that the lack of hemozoin inhibition by eQN and eQD is due to altered monomeric heme binding arising from the change in configuration at the 9-position carbon. Consistent with solution and solid-state nuclear magnetic resonance (NMR) data, Alumasa and coworkers recently suggested that QN forms a highly
fluorescent 1:1 complex with FPIX through a dative Fe—O interaction which is stabilized by an intramolecular five-membered ring formed via hydrogen bonding between the hydroxyl proton and quinuclidine nitrogen (Alumasa et al. 2011). Alumasa et al. also showed that, in solution, QN and QD bind to monomeric FPIX with high affinity and perturb the FPIX monomer-dimer equilibrium in favor of monomer (Alumasa et al. 2011).

In collaboration with my colleague, Dr. Alexander Gorka, we performed similar in vivo and in vitro experiments with the isomeric pairs to further elucidate Cinchona alkaloid antiplasmodial activity and resistance. We also measured the cytostatic (IC$_{50}$) and cytocidal (LD$_{50}$) activities for QN-susceptible (QNS) versus QNR strains, hemozoin inhibitory activities, heme binding affinity, magnetic moment for drug-heme solutions, drug effects on pH-dependent heme aggregation, and heme ring current effects on bound drug. We isolated eQN- and eQD-heme complexes formed in aqueous solution and determined their stoichiometry and fluorescence properties. In sum, the data elucidate key differences in heme interactions for the isomeric pairs, suggest a novel model for eQN and eQD complexation with heme, and shed light on the relevance of heme interactions for the cytostatic versus cytocidal activities of the Cinchona alkaloids.
3.2 Results

3.2.1. Part 1: Developing a Cytocidal Assay with Trypan Blue is Difficult

In order to potentially distinguish live vs. dead parasites following staining with trypan blue, the erythrocyte plasma membrane must be permeabilized in order for the dye to come in contact with the potentially-damaged parasite membrane. Saponin is known to permeabilize the RBC membrane selectively (Siddiqui et al. 1979). In order to observe the potential uptake of
Trypan Blue, various concentrations of saponin were tested on CQS D10 parasites that had been treated with 1 or 250 μM CQ for 24 hours as a positive control for nonviable cells, as well as no CQ, as a negative control. 0.04% saponin (final concentration) for 5 minutes was chosen for future experiments, as the parasites were not observed to be extra-erythrocytic (lysing was avoided), but were observed to be stained by 0.04% trypan blue in the positive control. Minimal staining was observed in the negative control. Examples of unstained and stained parasites are shown in Figure 3.1.

![Unstained and Stained Parasites](image)

**Figure 3.1.** Microscopic Images of Unstained and Stained Parasites.

After confirming the optimum permeabilization conditions, the incubation time of the trypan blue staining was optimized to ensure that viable cells were not stained due to potential toxic effects of the dye itself. Untreated Dd2 parasite culture was permeabilized and stained with trypan blue. At every 5 minute time point between 0 and 30 minutes after staining, the numbers of stained and unstained parasites were counted (as many as possible in 5 minutes, but always more than 50).
The staining of viable parasites increases over the period, as shown in Figure 3.2. In order to minimize unwanted staining, the stain would be added to the sample and immediately processed by obtaining color images of bright-field microscopy under 100x magnification, see Figure 3.3. Parasites within the images can then be counted, stained vs. unstained. Treating Dd2 parasites with LD$_{50}$ CQ (21.6 µM) for 6 hours yields images like that shown in Figure 3.3, which also contains untreated (no CQ) and 5xLD$_{50}$ (180 µM) for 24 hours as negative and positive controls, respectively.

**Figure 3.2.** Staining of Untreated Parasites by Trypan Blue Over Time.
Figure 3.3. Representative Images of Trypan-Blue Stained Parasites. Parasites circled in blue are stained, while parasites circled in red are not.
The percentage of parasites stained vs. the calculated LD dose (0 μM [no drug], 50 μM for 6 hour LD₅₀ dose, 100 μM for 24 hour 5xLD₅₀), can be plotted, as shown in Figure 3.4. The plot is fairly linear, but the variation between percentage stained in different fields of view results in high levels of error. Additionally, the no-drug negative control shows high levels of background staining, suggesting that an assay based on this approach would have poor signal-to-noise ratios.

**Figure 3.4.** Change in Percentage of Parasites Stained Versus Lethal Dose Value. Each point is the average of 3 independent fields of view, with the error bars representing the standard error of the mean, 3 determinations total.
In light of these results, our laboratory abandoned this approach and focused on another, SYBR Green I dye-based approach that proved successful (Paguio et al. 2011), see “Discussion” and Chapter VII, section 7.3.

3.2.2. Part 2: Hz is not a Likely CQ Cytocidal Target.


The broad FPIX absorbance peak observed in the absence of SDS, characteristic of the μ-oxo dimer (Casabianca et al. 2008), exhibits maximum absorbance (λ max) at 389 nm (Fig. 3.5A, arrow 2), with a shoulder at 359 nm (Fig. 3.5A, arrow 1). My colleagues Drs. Gorka and Alumasa found that simple titration of the μ-oxo dimer with SDS results in a red shift of the FPIX Soret band from 389 nm to 405 nm (Fig. 3A, arrow 3), as well as a concentration-dependent increase in absorbance. This is presumably because upon addition of SDS at concentrations above the critical micelle concentration, free μ-oxo dimeric heme is converted to its monomeric form (Casabianca et al. 2009), generating the λ max shift to 405 nm and a concomitant increase in the extinction coefficient to 99,690 (M⁻¹ cm⁻¹). This effect is also easily measured in 96-well plate format (Fig. 3.5B), and the linear increase in absorbance then allows
for calibration of free FPIX at a known SDS concentration. That is, conversion of FPIX to Hz can be quantified via simple addition of SDS, without plate-washing steps.

**Figure 3.5.** (A) SDS Effects on the Absorbance Spectrum of FPIX. Increasing amounts of SDS were titrated into a cuvette containing 10 μM FPIX while the absorbance spectrum was recorded after each addition. Results are shown for no SDS (red), 0.1% (3.47 mM) SDS (brown), 0.5% (17.3 mM) SDS (green), 1% (34.7 mM) SDS (black), 2.5% (86.7 mM) SDS (blue), and 5% (173 mM) SDS (pink). (Inset) Peak height at 405 nm versus SDS concentration. a.u., absorbance
units. (B) Effect of SDS on the concentration-dependent absorbance (Abs.) of FPIX in the 96-well plate format. Results are shown for no SDS (●, $R^2 = 0.996$), 0.1% (3.47 mM) SDS (○, $R^2 = 0.988$), 0.5% (17.3 mM) SDS (▼, $R^2 = 0.992$), 1% (34.7 mM) SDS (■, $R^2 = 0.996$), 2.5% (86.7 mM) SDS (□, $R^2 = 0.998$), and 5% (173 mM) SDS (Δ, $R^2 = 0.999$). Increasing the concentration of SDS increases the absorbance of FPIX to a maximum at ~2.5% SDS. Each data point is an average of three independent measurements, each performed in triplicate (9 determinations total). Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)

An assay was previously developed in the Roepe Lab that takes advantage of this observation and that also mimics formation of Hz in vivo at physiological temperature, pH, and ionic strength. The Hz-crystallization catalytic abilities of various lipids that are suggested from recent reports to reside within the DV, were systematically compared to the catalytic efficiency of PC and of other lipids, as well as mono- or diacyl glycerols (Fig. 3.6) (Tripathi et al. 2002; Jackson et al. 2004). At physiological temperature, catalysis by PC was comparable to if not better than that found for all other lipids tested, including a blend of PC, pMAG, and sMAG (Fig. 3.6, BD). The lipid-catalyzed formation of Hz is pH dependent, with the strongest pH dependence found for PS and the weakest found for PC and the PC-pMAG-sMAG blend. More acidic aqueous conditions favor formation of the heme head-to-tail dimer (Egan et al. 2001); thus, acceleration of Hz formation at lower pH is expected.
Figure 3.6. Optimization of Lipid Catalyst. Comparison of the Hz (β-hematin) catalytic efficiency of PS (1-α-phosphatidyl-l-serine), SG (1-monostearin, sMAG), DO (1,2-dioly-rac-glycerol [18:1, cis-9]), DT (1,2-dioctanoyl-sn-glycerol-3-PC), PG (1-monopalmitin, pMAG), DG (1,2-dipalmitoyl-sn-glycerol-3-PC), PC (1-α-phosphatidylcholine), and BD (blend, meaning an equimolar mixture of PC, pMAG, and sMAG resulting in 1 mM/well final concentration). FPIX (100 μM) was incubated in 1 M propionate buffer (at the different pH values) at 37°C in the presence of 1 mM the corresponding lipid. The amount of Hz formed in the corresponding lipid wells (average of triplicate wells) was quantified relative to that for a lipid-free control after a 16-h incubation period. The 100% Hz yield was calculated via equation 1 in Material in Methods, with the sample absorbance being that of 100 μM FPIX. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)
PC was chosen as the catalyst for additional work because it performed similarly to the blend yet is less expensive and more easily handled. The optimal FPIX concentration was determined to be 100 μM, since this provides a wide dynamic range (>0.6 absorbance unit), as it is converted to Hz over approximately 16 h. Also, under control assay conditions, conversion of 100 μM FPIX to Hz was near 100%. Again, PC was found to be the most efficient catalyst at either pH (Fig. 3.7; Table 1), and Hz formation appeared to plateau at about 16 h. For the initial rates of Hz formation at pH 5.2, PC catalysis was 1.5-, 2-, and 5-fold faster than that with pMAG, sMAG, and the control (no lipid), respectively, and that the rate for PC-catalyzed Hz formation in this assay was very similar to that defined for the rate of Hz growth in live parasites (Pisciotta et al. 2007).
Figure 3.7. pH-Dependent Kinetics for the Production of Hz (β-hematin) in the presence of PC (●), pMAG (○), or sMAG (▼) or the absence of a catalyst (Δ). Sonicated lipids were incubated at 37°C with 100 μM FPIX in 200 μL of propionate buffer for 16 h at pH 5.2 (A) and pH 5.6 (B). Curve fitting was performed with SigmaPlot (version 10.0) software using the equation y = a(1 − bx). (Insets) The corresponding initial rate plots from time zero (t₀) to 4 h (t₄) (Table 3.1). Inset data are fit to linear regressions, and the initial rates were extracted from the slopes. Each data point is an average of three independent measurements, each performed in triplicate (9 determinations total).
Table 3.1. Initial rates of production of Hz in the presence of PC, pMAG, and sMAG and in the absence of lipid catalysts. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Initial rate(^a) (M s(^{-1}) ([10^{-8}]))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.2</td>
</tr>
<tr>
<td>No lipid</td>
<td>0.67</td>
</tr>
<tr>
<td>PC</td>
<td>3.61</td>
</tr>
<tr>
<td>pMAG</td>
<td>2.39</td>
</tr>
<tr>
<td>sMAG</td>
<td>1.78</td>
</tr>
</tbody>
</table>

\(^a\)Initial rates were calculated within the first 4 h of the reaction (see insets in Fig. 3.7).

Using this standardized assay, the inhibition of Hz formation by common antimalarials (CQ, QN, QD, and AQ) was quantified at several pHs. Overall, similar to other studies (Dorn et al. 1998; Huy et al. 2007, Ncokazi et al. 2005), we found that potencies were AQ > CQ ≈ QD > QN across the range of pHs (Fig. 3.8). We noted that different absolute IC\(_{50}\)s were obtained at the same pH when the identity of the lipid catalyst was changed (Table 3.2) but that the trends for the drugs remained similar. For CQ and AQ, the differences observed when PC versus pMAG catalysts were used were relatively small (2- to 3-fold); however, interestingly, QN and QD IC\(_{50}\)s were more perturbed by altering the lipid composition. This is consistent with the differences in CQ versus QN lipid partitioning behavior noted earlier (Casabianca et al. 2009).
Figure 3.8. Effect of pH on the Hz Inhibition ($\beta$-hematin inhibitory activity [BHIA]) IC$_{50}$ of known antimalarial drugs: CQ (●), AQ (○), QN (▼), and QD (Δ). Experiments were performed at 37°C in a 16-h assay. Each data point (± SD) is the average of three independent measurements, each performed in triplicate (9 determinations total) at the corresponding pH. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)

It has previously been suggested that Hz inhibition and antiplasmodial potency are well correlated (Dorn et al. 1998). However, another study observed that upon correction for different levels of DV accumulation, the linear correlation existed only for CQS strains (Hawley et al. 1998). In both of these studies, the nonphysiological Hz formation conditions and the wide structural diversity of the examined drugs complicate interpretation. We thus tested for a possible correlation between inhibition and antiplasmodial activity using a series of CQ analogues, as described in detail in Chapter II- Materials and Methods, section 2.2.3. I synthesized compounds
6-9 used in this analysis. These compounds were added to the set of analogs because they range in side-chain chemistry including: primary, secondary, and tertiary amines that are either diprotic or triprotic, and with alcohol and carbamate functional groups. Combined with additional CQ analogs, these compounds are structurally similar yet span a wide range of antiplasmodial IC$_{50}$s and LD$_{50}$s (Scheme 3.1; Table 3.3) (Natarajan et al. 2008; Ekoue-Kovi et al. 2009; Iwaniuk et al. 2009; Yearick et al. 2008). These analogues also span the effective monobasic, dibasic, and tribasic character and thus diverge in calculated VARs (see Materials and Methods). When Hz inhibition for these compounds was plotted against the antiplasmodial IC$_{50}$ determined versus live merozoite culture, we observed a good correlation when antiplasmodial activity for either CQS ($R^2 = 0.653$) or CQR ($R^2 = 0.639$) strains was tabulated (Fig. 3.9A and 3.9B). However, importantly, when LD$_{50}$ was plotted versus Hz inhibition, these correlations vanished (Fig. 3.9C and 3.9D).

Table 3.2. IC$_{50}$ for Hz inhibition at pH 5.2 for common antimalarials in the presence of PC, pMAG, or sMAG. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)

<table>
<thead>
<tr>
<th>Compound</th>
<th>BHIA IC$_{50}$ (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>CQ</td>
<td>67.7</td>
</tr>
<tr>
<td>AQ</td>
<td>25.4</td>
</tr>
<tr>
<td>QN</td>
<td>237</td>
</tr>
<tr>
<td>QD</td>
<td>111</td>
</tr>
</tbody>
</table>

$^a$PC, pMAG, or sMAG were present at 1 mM. IC$_{50}$s were determined using a 16-h endpoint assay at 37°C. BHIA, β-hematin inhibitory activity.
**Figure 3.9.** Correlation Plots Between the Hz inhibition (β-hematin inhibitory activity [BHIA]) IC\(_{50}\) and Antiplasmodial Activities for CQ analogs against HB3 (A, C) or Dd2 (B, D) for a series of CQ analogues. Best-fit plots of the data were generated by the least-squares method using SigmaPlot software. (A) Plot of BHIA IC\(_{50}\) versus antiplasmodial IC\(_{50}\) for CQS HB3 parasites (\(R^2 = 0.653\)); (B) plot of BHIA IC\(_{50}\) versus antiplasmodial IC\(_{50}\) for CQR Dd2 parasites (\(R^2 = 0.639\)); (C) plot of BHIA IC\(_{50}\) versus antiplasmodial LD\(_{50}\) for HB3 parasites (\(R^2 = 0.015\)); (D) plot of BHIA IC\(_{50}\) versus antiplasmodial LD\(_{50}\) for Dd2 parasites (\(R^2 = 0.057\)). IC\(_{50}\) and LD\(_{50}\) values are those found in Table 3.3. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a).
Table 3.3. Physical and pharmacokinetic properties, antiplasmodial IC$_{50}$ and LD$_{50}$, and BHIA IC$_{50}$ for antimalarial drugs and drug analogues used in this work (See Scheme 3.1). Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013a)

<table>
<thead>
<tr>
<th>Cpd IC</th>
<th>MW (g/mol)</th>
<th>pKa</th>
<th>ClogP</th>
<th>Diffusion Coefficient (x 10$^{-6}$ cm$^2$/s)</th>
<th>Antiplasmodial IC$_{50}$ (nM)</th>
<th>Antiplasmodial LD$_{50}$ (µM)</th>
<th>BHIA IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ</td>
<td>318.19</td>
<td>7.65 9.86</td>
<td>4.65</td>
<td>5.00</td>
<td>19.6 (0.4)</td>
<td>201.5 (9.1)</td>
<td>0.12 (0.01)</td>
</tr>
<tr>
<td>QN</td>
<td>324.42</td>
<td>5.69 9.10</td>
<td>3.26</td>
<td>5.00</td>
<td>107.2 (12.4)</td>
<td>265.6 (14.6)</td>
<td>9.1 (0.4)</td>
</tr>
<tr>
<td>QD</td>
<td>324.42</td>
<td>5.69 9.10</td>
<td>3.26</td>
<td>5.00</td>
<td>41.9 (6.0)</td>
<td>174.1 (3.1)</td>
<td>0.43 (0.1)</td>
</tr>
<tr>
<td>AQ</td>
<td>355.86</td>
<td>5.84 9.88</td>
<td>4.09</td>
<td>4.82</td>
<td>10.0 (3.3)</td>
<td>27.7 (2.9)</td>
<td>0.04 (0.007)</td>
</tr>
<tr>
<td>1</td>
<td>294.95</td>
<td>5.11 8.59</td>
<td>4.58</td>
<td>5.26</td>
<td>9530.0 (39.5)</td>
<td>5500.0 (28.2)</td>
<td>79.9 (0.1)</td>
</tr>
<tr>
<td>2</td>
<td>305.9</td>
<td>7.50 9.81</td>
<td>4.34</td>
<td>4.99</td>
<td>6.0 (0.7)</td>
<td>26.0 (3.5)</td>
<td>0.23 (0.01)</td>
</tr>
<tr>
<td>3</td>
<td>319.9</td>
<td>7.56 9.84</td>
<td>4.82</td>
<td>4.82</td>
<td>12.0 (0.9)</td>
<td>199.0 (11.1)</td>
<td>3.8 (0.1)</td>
</tr>
<tr>
<td>4</td>
<td>363.92</td>
<td>7.48 9.58</td>
<td>5.16</td>
<td>4.45</td>
<td>95.0 (10.6)</td>
<td>169.0 (16.2)</td>
<td>7.1 (0.1)</td>
</tr>
<tr>
<td></td>
<td>Calcd using SPARC online calculator (sparc.chem.uga.edu/sparc/liqpka/index.cfm?ionize=N13). pK(\text{as}) are for the quinolinyl and side chain aliphatic nitrogens, respectively, with the former listed from left to right beginning at the quinoline branch point (see Figure 3.7).</td>
<td>Calculated for octanol/water using the SPARC online calculator.</td>
<td>Calculated for water diffusion using the SPARC online calculator.</td>
<td>IC(50) values are an average of three independent trials, each performed in triplicate (9 replicates total). SEM is shown in parentheses.</td>
<td>LD(50) values are an average of two independent trials, each performed in triplicate (6 replicates total). SEM is shown in parentheses.</td>
<td>Paguio et al. 2011</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>362.9</td>
<td>7.70</td>
<td>9.35</td>
<td>3.15</td>
<td>4.48</td>
<td>79.0 (5.9)</td>
<td>94.0 (10.7)</td>
</tr>
<tr>
<td>6</td>
<td>249.74</td>
<td>7.65</td>
<td>10.04</td>
<td>2.44</td>
<td>5.97</td>
<td>2990.0 (31.8)</td>
<td>2390.0 (33.4)</td>
</tr>
<tr>
<td>7</td>
<td>292.92</td>
<td>7.44</td>
<td>9.17</td>
<td>4.4</td>
<td>4.46</td>
<td>&gt; 10000</td>
<td>3720.0 (42.5)</td>
</tr>
<tr>
<td>8</td>
<td>451</td>
<td>7.64</td>
<td>8.05</td>
<td>4.39</td>
<td>4.13</td>
<td>1680.0 (23.5)</td>
<td>6970.0 (51.2)</td>
</tr>
<tr>
<td>9</td>
<td>350.89</td>
<td>7.64</td>
<td>8.99</td>
<td>9.55</td>
<td>1.4</td>
<td>4.82</td>
<td>1390.0 (19.5)</td>
</tr>
</tbody>
</table>
As described earlier (Natarajan et al. 2008), replacement of the anilinal N by S or O for some of these compounds significantly lowers the quinolinial N pKa (to values near those seen for QN or QD; see Natarajan et al. 2008). In vivo antiplasmodial IC₅₀s are presumably defined, in part, by the fold accumulation of the drug at the site of Hz formation (i.e., the acidic DV interior). We therefore scaled antiplasmodial IC₅₀s and LD₅₀s by multiplying by the calculated VARs for each molecule, such that the in vivo IC₅₀s were normalized to predicted vacuolar accumulation (see Materials and Methods). However, this did not improve the correlation between either IC₅₀ or LD₅₀ and Hz inhibition (data not shown).

LD₅₀ versus IC₅₀ data highlight interesting trends for the cytocidal and cytostatic activities observed in the correlation analysis (Fig. 3.9). For CQ, CQR strain Dd2 was ∼10-fold resistant by IC₅₀ but ∼140-fold resistant by LD₅₀ (Paguio et al. 2011) (Table 3.3). In contrast, AQ has low-nM LD₅₀s, with no significant differences for HB3 versus Dd2 (Paguio et al. 2011). Interestingly, for compounds 1 and 7, Dd2 is hypersensitive by IC₅₀ (more sensitive than CQS strain HB3) but is either slightly resistant relative to HB3 (compound 1) or has sensitivity similar to that of HB3 (compound 7) when potency is quantified by LD₅₀. Dd2 is hypersensitive to compounds 6 and 9 by LD₅₀ but is resistant to compounds 2 to 5 and 8. Interestingly, some compounds with high IC₅₀s (low cytostatic activity) are more active by LD₅₀ (high cytocidal potency) (compounds 7 to 9). Overall, then, the good correlation between Hz inhibition and IC₅₀, with a lack of a correlation between Hz inhibition and LD₅₀ for the same drugs, spans a range of CQ compound behavior for CQS versus CQR strains.
3.2.3. Part 3: Hz is Not Likely to be a QN Cytocidal Target

Previously published as:


*eQN and eQD were prepared by Dr. Alexander Gorka via a one-pot Mitsunobu esterification-saponification reaction, as described previously (Sidorowicz *et al.* 2011). QN and QD were converted to the *p*-nitrobenzoate ester using *p*-nitrobenzoic acid and triphenylphosphine (oxidized to triphenylphosphine oxide via DIAD), followed by in situ saponification with lithium hydroxide, to afford eQN and eQD, respectively and purified (see Material and Methods).*

Cytostatic and cytocidal antiplasmodial activities of the compounds shown in Scheme 3.2 were determined for HB3 (QNS), Dd2 (QNR), and K76I (QN-hypersensitive) strains of *P. falciparum* using the SYBR green I assay (Bennet *et al.* 2004b; Paguio *et al.* 2011) (Table 3.4) by Dr. Alexander Gorka. Previously, strain K76I was found to be similarly CQR and QD resistant (QDR) relative to Dd2 (as defined by IC$_{50}$ data) yet hypersensitive to QN, owing to a PfCRT mutation at position 76 (Dd2 harbors a K76T mutation) (Cooper *et al.* 2002; Griffin *et al.* 2012). Similar to previous work (Karle *et al.* 1992), we found that eQN has an IC$_{50}$ ~60-fold
higher than that of QN against HB3 and ∼10-fold higher than that of QN against Dd2. The IC$_{50}$ of eQN for K76I was ∼70-fold higher than that of QN, behavior that is more similar to that against HB3 than to that against Dd2. For eQD, the effects were more pronounced, with IC$_{50}$ activities ∼190-fold and ∼25-fold higher than those of QD against HB3 and Dd2, respectively. For K76I, eQD had an IC$_{50}$ ∼10-fold higher than that of QD, behavior that is now more similar to that against Dd2 than to that against HB3 (e.g., the converse of the trend for eQN versus QN). Curiously, and as also found previously (Karle et al. 1992), Dd2 showed mild hypersensitivity to both eQN and eQD, and we found that relative to the sensitivity of HB3, strain K76I was even more hypersensitive to both 9-epimers than Dd2.

Table 3.4. Antiplasmoidal IC$_{50}$s and LD$_{50}$s for QNS strain HB3, QNR strain Dd2, and the K76I strain$^d$Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Experimental IC$_{50}$ (nM)$^a$</th>
<th>Experimental LD$_{50}$ (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HB3</td>
<td>Dd2</td>
</tr>
<tr>
<td>QN</td>
<td>107.2</td>
<td>(12.4)</td>
</tr>
<tr>
<td>eQN</td>
<td>6,588.5</td>
<td>(358.2)</td>
</tr>
<tr>
<td>QD</td>
<td>41.9</td>
<td>(6.0)</td>
</tr>
<tr>
<td>eQD</td>
<td>7,893.6</td>
<td>(46.8)</td>
</tr>
</tbody>
</table>

$^a$Experimental IC50 and LD50 values are averages of three independent measurements (9 replicates total), with SEMs reported in parentheses.

$^b$ $R_f = Dd2$ value/HB3 value.

$^c$ $R_f = K76I$ value/HB3 value.

$^d$ The K76I strain is hypersensitive to QN but resistant to QD (Cooper et al. 2002; Griffin et al. 2012; Cooper et al. 2007).
Importantly, however, these trends did not hold when potency was tabulated via cytocidal activity ($LD_{50}$; Table 3.4). eQN showed $LD_{50}s \sim 1.8$-fold and $\sim 1.2$-fold higher than those of QN against HB3 and Dd2, respectively. eQD $LD_{50}s$ were $\sim 40$-fold and $\sim 1.2$-fold higher than those of QD against HB3 and Dd2, respectively. Put another way, for any individual strain, the altered stereochemistry of the 9-epimers vastly decreased the cytostatic potency (changes $IC_{50}$) but (with one important exception for the $IC_{50}$ of eQD versus that of QD against HB3) only mildly altered the cytocidal potency ($LD_{50}$). One interpretation is that the molecular targets for Cinchona alkaloid cytostatic and cytocidal activities must differ. Also, on the basis of the patterns for the QNR strains versus those for the hypersensitive strains, it also seems likely that mechanisms of cytostatic versus cytocidal resistance must differ.

Consistent with these conclusions, when defined by $IC_{50}$, strain K76I exhibited rather significant ($\geq 8$-fold) hypersensitivity to eQN and QN relative to that of HB3 but similar ($< 2$-fold) differences in $LD_{50}s$ for the same drugs relative to those for HB3 (Table 3.4). The QD-eQD pair displays a different trend. Relative to HB3, strain K76I showed mild ($\sim 3.5$-fold) eQD hypersensitivity, as defined by $IC_{50}$, but 3-fold greater resistance, as defined by $LD_{50}$. Strain K76I was 5-fold more resistant to QD than HB3 via $IC_{50}$ but an incredible 200-fold more resistant than HB3 via $LD_{50}$. See also references Cooper et al. 2002; Griffin et al. 2012; Cooper et al. 2007; Sidhu et al. 2002; and Paguio et al. 2011 for selected quinoline activity data versus these strains.

As mentioned above, inhibition of parasite hemozoin formation is believed to be the principal basis of antiplasmodial activity for quinoline drugs like the Cinchona alkaloids. However, this theory is based entirely on cytostatic activity ($IC_{50}$) data, since, with one exception
(Paguio et al. 2011), in vitro antiplasmodial activity is always routinely quantified via IC$_{50}$. Data produced by Dr. Alexander Gorka shows that QN and QD actively inhibit the formation of hemozoin under physiological conditions in the low- to mid-μM range at both pH 5.2 and 5.6, eQN and eQD do not, with inhibitory IC$_{50}$s being above several mM (Table 3.5). We use measurement at both pH 5.2 and 5.6 to mimic variable physiological conditions, since different DV pHs for sensitive (HB3) versus resistant (Dd2) strains have been measured in some studies (Bennet et al. 2004a). These data suggest that the loss of cytostatic activity for the 9-epimers is due to an inability to prevent hemozoin formation, as also suggested by Egan et al. (2004).

Table 3.5. β-Hematin inhibitory IC$_{50}$ data at pH 5.2 and 5.6$^a$ Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>β-Hematin IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.2</td>
</tr>
<tr>
<td>QN</td>
<td>255.4 (22.7)</td>
</tr>
<tr>
<td>eQN</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>QD</td>
<td>176.2 (4.9)</td>
</tr>
<tr>
<td>eQD</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

$^a$ Less than 10% inhibition is seen for the 9-epimers at 1 mM, leading to an estimated IC$_{50}$ above several mM. Experimental IC$_{50}$ and values are averages of three independent measurements (9 replicates total), with SEMs reported in parentheses.

We next plotted the hemozoin (β-hematin) inhibitory IC$_{50}$ (β-hematin inhibitory activity [BHIA]) against a series of QN analogues with variable cytostatic potency (Dinio et al. 2012) to test for possible correlations (Fig. 3.10). The antiplasmodial IC$_{50}$ was indeed mildly correlated
with the BHIA IC\textsubscript{50} across the series (R\textsuperscript{2} > 0.54). However, interestingly, the correlation vanished when LD\textsubscript{50} data were plotted (R\textsuperscript{2} < 0.01; Fig. 3.10, caption). Consistent with the data in Table 3.4, this further suggests that Cinchona alkaloid targets for cytostatic versus cytocidal activities differ.

**Figure 3.10.** Antiplasmodial Activity of a Series of QN analogues (Dinio et al. 2012). (A) BHIA IC\textsubscript{50} versus antiplasmodial IC\textsubscript{50}; (B) BHIA IC\textsubscript{50} versus antiplasmodial LD\textsubscript{50}. The IC\textsubscript{50}s shown are averages of three independent measurements, each done in triplicate (9 replicates total) as reported previously (Dinio et al. 2012). LD\textsubscript{50} values are averages ± SEMs of three replicates and are as follows: CN-2, 2,337.9 ± 4.3 nM; CN-4, 2,360.6 ± 13.4 nM; QD-2, 2,234.1 ± 7.4 nM; QN-8, 2,251.4 ± 28.4 nM; QN-12, 2,346.5 ± 13.2 nM. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

As described below, the 9-epimers promote stabilization of monomeric heme. Ideally, experiments to determine the affinity of the isomeric pairs for binding monomeric FPIX would
be performed under physiologically relevant aqueous conditions. However, drug-heme titrations are performed in 40% aqueous DMSO due to the decreased intensity of the Soret band, less-than-strict adherence to Beer's law, insolubility, and potential for heme dimerization and aggregation outside these conditions (Egan et al. 1997; Casabianca et al. 2008; Collier et al. 1979). Dr. Alexander Gorka found that eQN binds monomer with a similar affinity as QN; however, eQD binds with a ~5-fold lower Ka than QD (Fig. 3.11; Table 3.6).

**Figure 3.11.** Drug-Heme Binding Curves. Affinity was measured by titrating increasing concentrations of QN (●), eQN (○), QD (▼), and eQD (Δ) into a solution of monomeric FPIX, followed by nonlinear least-squares analysis (Levenberg-Marquardt algorithm [Egan et al. 1997; Marques et al. 1996]) to determine Kₐ (Table 3.6; see Materials and Methods). a.u., absorbance units. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)
Table 3.6. Drug-heme affinity coefficients for binding monomeric FPIX\(^b\) Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Experimental $K_a$ for monomer ($\text{M}^{-1} [10^4]$)$^a$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>QN</td>
<td>0.75 (0.07)</td>
<td>1.00</td>
</tr>
<tr>
<td>eQN</td>
<td>0.61 (0.11)</td>
<td>1.00</td>
</tr>
<tr>
<td>QD</td>
<td>4.40 (0.04)</td>
<td>0.99</td>
</tr>
<tr>
<td>eQD</td>
<td>0.94 (0.10)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^a$ Experimental $K_a$ values are averages of three independent measurements (9 replicates total), with SEMs reported in parentheses.

$^b$ Drug-heme affinity coefficients for binding monomeric FPIX were obtained via nonlinear least-squares analysis of binding data using the Levenberg-Marquardt algorithm, which is described elsewhere (Egan et al. 1997; Marques et al. 1996). See the caption to Fig. 3.11 for binding data.

I studied the effect of various drugs on the pH-dependent solubility of monomeric FPIX by varying the pH versus a constant FPIX concentration (178 $\mu$M) and drug concentration (20 $\mu$M). Previous studies of the pH-dependent aggregation of FPIX, and the effect of quinolines on this phenomenon determined that the effect of quinolines on the aggregation of FPIX does not depend on the identity of the quinoline, for example CQ and AQ result in the same shift of pH-dependent solubility. The alteration is also not stoichiometric, suggesting an as-yet not fully defined nucleation phenomenon, where variations of drug concentration up to 8 orders of magnitude do not impact the shift in solubility (Ursos et al. 2001). We found that eQN and eQD did not affect the pH-dependent solubility of FPIX, whereas QN, QD, CQ, and AQ produced a marked effect over a relatively narrow pH range (Fig. 3.12A; see also Ursos et al. 2001). This effect is immediately apparent when the midpoints of each solubility curve are quantified (Fig. 3.12B). Just as QN and QD differ from their 9-epimers in their inhibition of Hz, a difference is
seen in the effect on aggregation of FPIX. Together, these observations suggest that the alteration of the stereochemistry of QN and QD alters the compound’s ability to interact with FPIX and prevent Hz formation in \textit{P. falciparum} parasites in a manner that distinguishes them from other studied quinolines, including CQ and AQ.

\textbf{Figure 3.12.} Drug Effects on pH-Dependent Heme Aggregation. (A) Concentration of free (nonaggregated) FPIX remaining in solution versus pH in the presence of no drug (●), CQ (○), QN (▼), QD (Δ), AQ (■), eQN (□), and eQD (♦); (B) the midpoint (pH$_{1/2}$) of each curve was calculated via sigmoidal regression. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka \textit{et al.} 2013d)

Dr. Alexander Gorka next isolated the eQN-, eQD-, QN-, and QD-monomeric FPIX complexes formed under aqueous conditions using methods published previously (Alumasa \textit{et al.} 2011) and characterized their properties. Qualitatively, we found that the 9-epimer adducts are weaker than those formed with QN and QD, as evidenced by the ease of resolubilization in
Figure 3.13. Fluorescence Excitation and Emission Spectra for 5 μM QN-FPIX adduct (gray dotted line, top), QD-FPIX adduct (gray dash-dot-dot line, second from top), QD (gray continuous line, third from top), QN (gray dashed line, fourth from top), eQD (black dash-dot line, fifth from top), eQN (black dotted line, sixth from top), eQN-FPIX adduct (black dash-dot-dot line, seventh from top), eQD-FPIX adduct (black continuous line, eighth from top), and FPIX (black dashed line, bottom) performed in 1:1 methanol–0.2 M HEPES (pH 7.2) at an excitation λ of 334 nm and an emission λ of 371 nm. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

neutral buffer following precipitation and the fact that yields were significantly lower than those for QN and QD under the same conditions. The eQN- and eQD-FPIX adducts were also ~20- and 10-fold less fluorescent, respectively, than the QN- and QD-FPIX adducts (Fig. 3.13; Table
Table 3.7. Drug-monomeric heme adduct properties. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)

<table>
<thead>
<tr>
<th>Species</th>
<th>Fluorescence maximum (10^3)a</th>
<th>Adduct stoichiometryb for drug-FPIX</th>
<th>ESI molecular ion(s) observed (m/z)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λex (334 nm)</td>
<td>λem (371 nm)</td>
<td></td>
</tr>
<tr>
<td>FPIX</td>
<td>1.13</td>
<td>1.15</td>
<td>616</td>
</tr>
<tr>
<td>QN-FPIX</td>
<td>360</td>
<td>363</td>
<td>325, 616, 938, 940, 941</td>
</tr>
<tr>
<td>eQN-FPIX</td>
<td>17.6</td>
<td>17.8</td>
<td>325, 616</td>
</tr>
<tr>
<td>QD-FPIX</td>
<td>255</td>
<td>256</td>
<td>325, 616, 938, 940, 941</td>
</tr>
<tr>
<td>eQD-FPIX</td>
<td>21.7</td>
<td>21.9</td>
<td>325, 616</td>
</tr>
</tbody>
</table>

a Data correspond to those in Fig. 3.13. λex and λem, excitation and emission λ's maxima, respectively.

b Values are averages of measurements performed in methanol, acetonitrile, and 40% (vol/vol) DMSO–0.2 M HEPES (pH 7.2), with SEMs reported in parentheses.

c m/z 325, (M + H)^+ for QN or QD free base; m/z 616, (M)^+ for FPIX; m/z 938, 940, and 941, (M − 2H)^+, (M)^+, and (M + H)^+, respectively, for C54H55FeN6O6, corresponding to a 1:1 drug-heme complex. Peaks at m/z 498, 771, and 860 were also observed and were possible FPIX and adduct fragments. See also Fig. 3.14.

3.7). ESI-MS analysis of the QN and QD complexes gave rise to peaks at m/z 325 and 616, corresponding to free QN H^+/QD H^+ and free FPIX monomer, respectively, and clear major peaks at m/z 938, 940, and 941 (Table 3.7; see Fig. 3.14). These findings coincide with the calculated m/z values of (M − 2H)^+ equal to 938.6, (M)^+ equal to 940.4, and (M + H)^+ equal to 941.4 for C54H55FeN6O6, corresponding to 1:1 drug-FPIX complexes (Alumasa et al. 2011). The 9-epimer adducts, however, did not survive electrospray ionization, as evidenced by peaks at m/z
325 and 616 only (Table 3.7; Fig. 3.14), which again suggests that these are drug-heme complexes weaker than those formed by QN and QD (Alumasa et al. 2011).

**Figure 3.14.** ESI Mass Spectra for A) QN-FPIX adduct, B) QD-FPIX adduct, C) eQN-FPIX adduct, and D) eQD-FPIX adduct. m/z 325: (M + H)+ for QN or QD free base, 616: (M)+ for FPIX, 938, 940, 941: (M – 2H)+, (M)+, and (M + H)+ for C_{54}H_{55}FeN_{6}O_{6}, corresponding to a 1:1 drug-heme complex. Peaks at m/z 498, 771, and 860 are possible FPIX and adduct fragments. Copyright © 2013, American Society for Microbiology. Used with Permission. (Gorka et al. 2013d)
3.3 Discussion

Parts of this section were previously published as:


Quantifying cytocidal activity typically relies on the ability to distinguish the difference between organisms that are alive, and those that are dead. One convenient method is the use of fluorescent or visible dyes that label based on normal physiological conditions or evidence of damage. In the case of *Plasmodia*, the organism resides within a host cell, isolating it from the extracellular dye. By permeabilizing the RBC membrane with saponin, it is possible to stain intraerythrocytic *P. falciparum* with the live/dead stain trypan blue. As seen in Figure 3.1, the stained versus unstained parasites can be identified when photographed under 100x magnification. By taking images of several fields of view, seen in Figure 3.3, it is possible to count up to 100 parasites within a short period of time, which is important as the dye itself is toxic. However, overcoming the two membranes that separate the parasite plasma membrane from the extracellular dye poses a procedural problem. The use of saponin to permeabilize the RBC membrane suffers from a tendency to lyse the membrane completely if the sample is
incubated too long or agitated too vigorously (data not shown). Additionally, the mere presence of trypan blue can alter staining of viable cells over time (Figure 3.2). Attempts to construct a standard curve of staining, using 5xLD$_{50}$ for 24 hours as a “LD$_{100}$”, results in a linear trend (Figure 3.4), but even when the sample is not treated with drug, approximately 50% of the parasites are observed to be stained. This high level of background staining, combined with the variability between sample preparations, and even fields of view of one sample, precludes its use in a cytocidal assay, as the dynamic range from no drug to toxic levels is not great enough to be used to plot a full growth curve. Additionally, counting cells with microscopy is not amenable to scaling to higher-throughput methods.

At about the same time as I was doing this work, Paguio et al. (2011) examined additional “live/dead” stains such as hydroethidine and carbocyanines, and they were found to also have low dynamic ranges and high levels of variability in staining. Instead of trying to utilize these stains, which suffer from low signal (hydroethidine) or high background (carbocyanines and trypan blue- see Figure 3.3), the widely-used cytostatic assay based on the fluorescence of DNA-intercalating SYBR Green I (Bennet et al. 2004b) was adapted for short-term bolus dosing, followed by washing the drug away and growth for 48 hours (one parasite life cycle). Using this assay, cytocidal activities were determined, and altered cytocidal resistance phenomenon were described. For example, the compound verapamil, which is known to chemosensitize Dd2 parasites to CQ at static levels, was determined to not alter the resistance to bolus-dose levels of resistance (no reduction in LD$_{50}$ value) (Paguio et al. 2011).

In order to further explore the difference between cytostatic and cytocidal drug effects, a more physiologically relevant high-throughput assay for quantifying inhibition of Hz formation
was developed, and examined for correlation with antiplasmodial activity. Consistent with most data from other Hz formation assays, Hz inhibition measured under physiological conditions decreases for all drugs examined upon decreasing pH. Presumably, more acidic conditions require a higher drug concentration to inhibit crystal growth because crystallization is accelerated and the solubility of precrystalline heme intermediates to which drugs bind is decreased at these pHs. We found that the efficiency of phosphotidylcholine in catalyzing Hz formation is comparable to if not better than the efficiency of catalysis by other lipids found in DV lipid nanospheres harboring nascent Hz crystals. It is currently not known specifically which of the lipids accelerates the Hz formation, but the data suggest that all known components of these nanospheres are excellent catalysts (Pisciotta et al. 2007; Jackson et al. 2004).

This assay was tested for correlations between Hz inhibition and the antiplasmodial IC₅₀ in order to ascertain if more close-to-physiological conditions for Hz formation modified those conclusions. Dorn et al. (1998) were the first to directly test the hypothesis that antiplasmodial activity was correlated with Hz inhibition by examining a small set of common antimalarials (quinoline methanols, 4-aminoquinolines, acridines, and nonquinolines). Although Cohen (1964) and Macomber and Spintz (1969) proposed that heme was a target for quinoline antimalarial drugs more than 30 years before this study, results from Dorn et al. (1998) have been crucial for exploring the molecular pharmacology of antimalarial drugs and have been highly influential in guiding additional antimalarial drug development. Using a CQS strain (NF54), a linear correlation was observed between the potency of Hz inhibition and antiplasmodial IC₅₀ for common antimalarials, now known as “heme-acting” antimalarial drugs. Hawley et al. (1998) further investigated this concept using CQS (3D7) and CQR (K1) strains and a similar series of
quinoline methanols, 4-aminoquinolines, and acridines, however, these authors observed no apparent correlation between the two activities when comparing raw IC$_{50}$ data for either CQS or CQR strains. After using a VAR correction, Hawley and coworkers (1998) did observe a correlation for the CQS strain only. A third study by Kaschula et al. (2002), utilizing a series of 2-carbon 4-aminoquinoline derivatives with variable functionalities at position 7, observed a correlation between the two activities, but again, only when the antiplasmodial IC$_{50}$ was scaled to the relative VAR. There are logical explanations for these differing results. First, the use of a nonhomologous series of compounds (e.g., quinolines versus acridines) with very different bioavailability profiles, as well as the nonphysiological conditions of the Hz formation assays that were used, makes interpretation more complex. There are multiple Hz precursors (FPIX monomer, μ-oxo dimer, and head-to-tail dimer) against which this range of pharmacophores interact (de Dios et al. 2004; de Dios et al. 2003; Leed et al. 2002; Casabianca et al. 2008; Casabianca et al. 2009; Alumasa et al. 2011), and the relative effectiveness of monomeric heme-drug versus dimeric heme-drug complexes for inhibiting Hz formation likely differs.

A complete understanding of the correlation between antiplasmodial IC$_{50}$ activity and Hz inhibition requires more detailed chemical information on the nature of solid-state aggregates of heme and quinoline compounds. Earlier studies clearly showed that the solubilities of monomeric and dimeric forms of FPIX are dramatically affected by the presence of quinolines (Casabianca et al. 2008; Ursos et al. 2001), that CQ and other quinoline antimalarials bind with different affinities to monomeric versus dimeric FPIX (de Dios et al. 2003; Leed et al. 2002; Casabianca et al. 2008), and that the solubility of drug-heme complexes in hydrophobic phase may be more relevant to Hz inhibition potency (Pisciotta et al. 2007; Casabianca et al. 2009).
Taken together, these data show that quinoline drug-heme interactions are significantly more complex than simple aqueous equilibria predict.

Regardless, the principle conclusion of Dorn et al. (1998) has continued to strengthen the notion that the site of quinoline antimalarial drug action must be the DV, where the drug then binds to free heme to prevent Hz crystallization. Altered DV biochemistry then reduces drug-heme interaction and causes resistance to these drugs, a prediction that has been validated by studies on the function of mutant \textit{P. falciparum} chloroquine resistance transporter (PfCRT; a DV membrane protein), which causes an elevated CQ IC$_{50}$ in \textit{P. falciparum} malaria (Roepe 2011).

A good correlation was observed between antiplasmodial IC$_{50}$s and Hz inhibition across a series of CQ analogues with a wide range of activities (Fig. 3.9), similar to the conclusions of Dorn et al. (1998). Using Hz inhibition quantified under physiological conditions with lipid catalyst, the correlation is not necessarily improved when estimated VARs, or other pharmacokinetic properties (Table 3.3), are applied to the data set. Regardless, although the IC$_{50}$s of our set of CQ analogues span a very wide range, they are not necessarily representative of all CQ analogues studied to date, and correlation between antiplasmodial IC$_{50}$ and Hz inhibition IC$_{50}$ for quinoline antimalarials may be dependent (at least in part) upon the specific drugs that are assessed.

Critically, however, and in distinct contrast to the correlation with the IC$_{50}$, there is a complete lack of correlation between the Hz inhibition IC$_{50}$ and antiplasmodial LD$_{50}$ for the same series of CQ analogues (Fig. 3.9C and D). The test series of CQ analogues were chosen because they exhibit a wide range of cytostatic and cytocidal activities (Table 3.3) and also show a variety of other interesting trends. Strain Dd2 resistance to CQ is 10-fold by IC$_{50}$ but 140-fold
by LD$_{50}$, suggesting that a significantly different pharmacology may be relevant to understanding the different modes of action for CQ (Paguio et al. 2011). AQ does not exhibit significant differences in IC$_{50}$ and LD$_{50}$ activity for HB3 versus Dd2 and is quite potent (low nM) by both antiplasmodial measurements (Table 3.3). This suggests to us that the cytocidal targets for CQ versus AQ may differ and/or that the mechanism of CQ cytocidal resistance is distinct from that for AQ.

With these results, we suggest that three critical principles are becoming increasingly apparent: (i) potent IC$_{50}$ activity can but does not always indicate potent LD$_{50}$ activity for quinoline antimalarials, (ii) resistance to cytostatic drug effects does not always correlate with resistance to cytocidal drug effects, and (iii) for CQ and CQ analogues, the ability to inhibit Hz formation correlates strongly with IC$_{50}$ potency but not with LD$_{50}$ potency. The large differences between IC$_{50}$ and LD$_{50}$ measures of activity against HB3 and Dd2 parasites suggest that the molecular mechanisms of resistance to the cytostatic and cytocidal effects of quinoline antimalarial drugs likely differ (see Cabrera et al. 2009a). For CQ, cytostatic resistance (elevated IC$_{50}$) is mediated by PfCRT and/or P. falciparum multidrug resistance protein (PfMDR1) mutations that alter the electrochemical potential-driven DV transport of the protonated drug (Roepe 2011; Cooper et al. 2002; Cooper et al. 2007; Ferdig et al. 2004; Sidhu et al. 2005; Sidhu et al. 2002) and, hence, access to heme. However, mounting experimental evidence (Paguio et al. 2011; Cabrera et al. 2009a; Gaviria et al. 2013) suggests the possibility of additional (non-DV) cytocidal targets for CQ, which then predicts the possibility of additional cytocidal resistance mechanisms that are mechanistically distinct from cytostatic resistance mechanisms (Sinai et al. 2012; Gaviria et al. 2013).
For quinoline methanols quinine and quinidine, the differences in fold resistance are less dramatic than CQ. At cytostatic levels, QN shows 2-fold resistance in Dd2 vs. HB3, while QD shows 7-fold resistance. At cytocal levels, the compounds are 4- and 60-fold resistant for Dd2 vs. HB3. Similar to Karle et al. (1992), we observed vast differences in IC₅₀ activity between QN and QD and their 9-epimers, with the epimers showing significantly reduced potency (Table 3.6). In contrast, the activities measured by LD₅₀ tell a different story. While eQN and eQD were less potent than QN and QD for each strain when measured by IC₅₀, the fold differences in LD₅₀ between the isomer pairs were far lower, and in some cases (e.g., strain K76I), the 9-epimer LD₅₀s were lower (they had higher cytocal potency than QN and QD). We found that relative to strain HB3, Dd2 was resistant to QN and hypersensitive to eQN when potency was defined via IC₅₀ but that the strain was resistant to both when potency was defined by LD₅₀. Overall, these observations and others lead to several major conclusions.

First, the large differences in IC₅₀s (but not LD₅₀s) observed for these isomers support the hypothesis that the molecular mechanisms of resistance to the cytostatic and cytocal effects of quinoline antimalarial drugs likely differ (see also Paguio et al. 2011), which is consistent with conclusions from previous drug transport analyses (Cabrera et al. 2009a). Cytostatic resistance to QN (i.e., elevated IC₅₀) is believed to be mediated at least in part by PfCRT and/or PfMDR1 mutations that alter electrochemical potential-driven DV transport of the protonated drug (Sidhu et al. 2005; Ferdig et al. 2004; Roepe 2011; Sidhu et al. 2002). A lack of eQN cytostatic resistance in Dd2 (lower IC₅₀ relative to that of HB3) is easily rationalized by the finding that stereochemically distinct eQN was not transported as well as QN by the Dd2 mutant PfCRT, since substrate recognition by transporters is often quite stereoselective (Roepe 2011).
We also suggest that the highly altered cytostatic activities but more similar cytocidal activities of the 9-epimers relative to those of QN and QD indicate that the stereochemistry of the 9-epimers negatively affects the interaction with the predominant cytostatic (IC\textsubscript{50}) target but does not necessarily affect the interaction with the predominant cytocidal (LD\textsubscript{50}) target(s). Consistent with the hemozoin inhibition data presented here, as well as in other studies (Egan \textit{et al.} 2005; Egan \textit{et al.} 1994; Leed \textit{et al.} 2002), we favor the interpretation that the predominant IC\textsubscript{50} target for quinoline antimalarials is one or more forms of uncrystallized heme within the DV, specifically, monomeric FPIX heme in the case of QN and QD (Alumasa \textit{et al.} 2011). This is consistent with the reduced hemozoin inhibition activity of the 9-epimers, and the lack effect on pH-dependent heme aggregation that is believed to facilitate hemozoin crystallization (Ursos \textit{et al.} 2001). The BHIA IC\textsubscript{50}s versus antiplasmodial activities for a series of five structurally related QN analogs synthesized previously (Dinio \textit{et al.} 2012) shows a strong correlation with cytostatic potency but not cytocidal potency, similar to the findings described above for CQ analogs. The 9-epimers do not inhibit formation of hemozoin under physiological conditions, and although they promote the high-spin FPIX monomer in solution (data not shown) and form complexes with FPIX that precipitate from aqueous solution, they do not do so with the same affinity, stoichiometry, and fluorescence properties as the complexes formed with QN and QD. The former are qualitatively weaker and less favored, as evidenced by the ease of resolubilization in neutral buffer following precipitation and significantly lower yield. Moreover, the 9-epimer complexes do not survive electrospray ionization, leading to sole peaks corresponding to free drug and FPIX in the mass spectrum (Table 3.7, Figure 3.14).
The 9-epimers also do not have a measurable effect on pH-dependent heme aggregation (Fig. 3.12). The potent cytostatic compounds, CQ, QN, QD, and AQ, all shift the midpoint of the pH-dependent solubility curve by about 0.15-0.2 pH units. By shifting the solubility curve, the aggregation of heme at a set pH is altered, which could effect the various equilibria associated with the hemozoin formation pathway (see Alumasa et al. 2011 for a full description); possibly this is a factor in the reduced Hz production observed in drug-treated parasites. (Gligorijevic et al. 2006a). This altered interaction with pre-crystalline heme species is consistent with the weakly-bound complex discussed previously, as well as the inability of eQN and eQD to inhibit hemozoin formation.

The recent revelation that there are altered patterns of resistance to antimalarials when used under cytostatic versus cytocidal conditions has resulted in a push to define the death pathway caused by antimalarial treatment and quantify its dynamics. The existence of two additional membranes between the parasite plasma membrane and the extracellular environment makes it difficult to use the traditional method of live/dead stains, both those that rely on membrane integrity (i.e. trypan blue), and those that rely on normal metabolic function and electrochemical differentials (i.e. hydroethidine and carbocyanines- see Paguio et al. 2011). However, the altered patterns of resistance have been quantified, via an adaptation of the traditional SYBR Green I cytostatic assay (Bennet et al. 2004b; Paguio et al. 2011).

Quantifying the cytocidal activity has allowed for examination of the correlation between in vitro measures of drug activity, such as the ability to inhibit the formation of β-hematin and the shift of the pH-dependent solubility curve of FPIX, and the drug’s activity. These in vitro methods that focus on heme and hemozoin correlate well with the cytostatic activities, both
across a series of CQ analogs and in the 9-epimers of quinine and quinidine. However, the association between β-hematin inhibition and activity at cytocidal activities is virtually non-existent. Similarly, although the 9-epimers of quinine and quinidine show little to no cytostatic activity, their cytocidal activities are within the same range as their parent compounds. It would seem that the heme crystallization pathway is a target of cytostatic quinoline drug treatment, but not cytocidal; more examination is needed to indentify the targets of cytocidal treatment.

3.4 Acknowledgements

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CHAPTER IV

SYNTHESIS, PURIFICATION, AND CHARACTERIZATION OF A NOVEL CHLOROQUINE PHOTOAFFINITY PROBE THAT UTILIZES CLICK CHEMISTRY FOR INVESTIGATING TARGETS IN P. FALCIPARUM PARASITES

This work to be published as:


4.1. Background

Resistance to chloroquine (CQR) in P. falciparum has been linked to multiple point mutations in the pfcrトgene that encode multiple amino acid substitutions for a digestive vacuole (DV) localized protein, Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT) (Fidock et al. 2000; Roepe 2011). The protein was initially hypothesized to impact resistance by either altering the pH of the acidic DV, thereby altering the accumulation of the dibasic drug, or through altering the transport of CQ across the vacuolar membrane (Fidock et al. 2000). A key prediction of the later is that PfCRT should bind CQ with meaningful affinity. Equilibrium binding studies and noted affinity constants for wild type and mutant (CQR-associated) PfCRT (Zhang et al. 2004) consistent with these conclusions. In addition, recently, direct evidence of CQ transport has been obtained (Paguio et al. 2009).

In order to further investigate PfCRT drug binding, and to hopefully map a CQ binding site within PfCRT, a probe, known as azido-biotinylated chloroquine (AzBCQ) (shown in Figure 4.1), was previously synthesized in our lab for use in the labeling and subsequent isolation of
target molecules, through the process known as “Activity-Based Protein Profiling” (ABPP) (Lekostaj et al. 2008; Salisbury et al. 2007).

![Figure 4.1. Structure of AzBCQ. Reprinted (adapted) with permission from (Lekostaj et al. 2008). Copyright (2008) American Chemical Society.](image)

This process, has been previously used to identify in vivo targets of small molecule probes. The probe must contain two reactive subunits: one which allows it to be covalently attached to the target, and another which is used for detection or purification. The probe can be either an irreversible enzyme inhibitor, or a reversible inhibitor labeled with a photo-active tag, such as a diazirine, an aryl azide, or benzophenone, which will covalently bind to biomolecules; both the target molecules and other molecules in the periphery (Salisbury et al. 2007). Labeling of the targets is typically followed by proteome analysis techniques. Typically, proteomes are investigated either through gel-based (single or two-dimensional separation) or chromatography-based (HPLC or avidin-affinity) techniques. Following separation of the identified proteins, mass spectrometry can be used for identification of the trypsin digestion products of the target proteins.
(Salisbury et al. 2007). ABPP allows for selectivity in proteomic methods that is not necessarily possible with global methods. Global methods examine the identities and relative levels of proteins from cell lysates, and are typically geared towards examination of proteins that are present in larger quantities. The use of separation methods and detection of probe-labeled proteins reduces background signal present from unrelated proteins, facilitating detection of proteins that may be in lower abundance (Best 2009). ABPP has been used to study the targets of small molecule inhibitors of enzymes such as human cytochrome P450s, serine hydrolases, proteasomes, and cathepsin proteases, as well as the targets of phenyl sulfonates, and enzymes that are poorly understood (Wright et al. 2007; Wright et al. 2009; Liu et al. 1999; Sletten et al. 2009; Speers et al. 2003; Mollering and Cravatt 2012).

In previous PfCRT studies, AzBCQ was derivatized with a photo-activated cross-linker, perfluorophenyl azide, as well as a biotin tag for purification (Lekostaj et al. 2008). The aryl azide photo-active label, after irradiation with ultraviolet light, will lose a molecule of dinitrogen to form a highly reactive nitrene, which can insert itself into a carbon-hydrogen bond, with the singlet nitrene more efficient at insertion than the triplet (Keana et al. 1990; Burdinski et al. 2006) as shown in Figure 4.2. In order to avoid ring-expansion reactions, which reduce the cross linking efficiency, aryl azides are typically perfluoronated, resulting in approximately 45% bond insertion. These perfluoronated aryl azides do absorb in the same UV region as most biopolymers, but the differences in molar absorptivity result in the activation of the probe with minimal biomolecular degradation (Keana et al. 1990).
Figure 4.2. UV-Activation of an Aryl Azide. A. Energy levels following UV-illumination are shown. B. An example of an aryl azide’s photochemistry is shown. Reprinted (adapted) with permission from (Burdzinski et al. 2006). Copyright (2006) American Chemical Society.
AzBCQ successfully labeled native PfCRT in saponin-isolated *Plasmodium falciparum* parasites, as well as partially purified PfCRT in proteoliposomes reconstituted from heterologous protein expressed in yeast (Lekostaj *et al.* 2008). Very efficient competition of the probe with underivitized CQ supports the idea that the AzBCQ binding site overlaps with the CQ binding site. The biotin tag allowed for isolation of the tagged protein on an SDS-PAGE gel, followed by visualization on a streptavidin blot, and subsequent mass spectral analysis of proteolysed PfCRT fragments. The mass spectroscopy analysis showed a labeled peptide comprised of amino acid residues 364-374. This region is predicted to be in an intra-DV loop between putative helices 9 and 10, and would contain a residue, 371, frequently mutated in CQR strains. Binding at this location leads to a hypothesized chloroquine binding pocket that would also involve helix 1, which contains the K76T mutation that is hypothesized to confer the CQR phenotype (Lekostaj *et al.* 2008).

ABPP has also been used previously by other groups to study binding in *P. falciparum*. Foley *et al.* iodine-125 labeled a CQ analog which is shown in Figure 4.3, with the iodine on the phenyl ring. This probe labeled 2 proteins (42 and 33 kDa). However, in this probe, the photoreactive group is directly attached via an amide bond to the aromatic pharmacophore, in the 7-position usually occupied by chlorine, potentially altering the electronic structure of the drug (Foley *et al.* 1994) and biasing drug-drug target binding. Additional studies of *P. falciparum* using ABPP included the study of possible inhibitors of cysteine proteases, which when inhibited impair the parasite’s ability to progress through the life cycle (Moellering and Cravatt 2012).
Figure 4.3. Structure of Photoaffinity CQ Probe Used by Foley et al. This research was originally published in *The Journal of Biological Chemistry*. Foley, M.; Deady, L. W.; Ng, K.; Cowman, A. F.; Tilley, L. Photoaffinity Labeling of Chloroquine-binding Proteins in *Plasmodium falciparum*. *The Journal of Biological Chemistry*. 1994, 269, 6955-6961. © the American Society for Biochemistry and Molecular Biology.

In order to detect proteins labeled during ABPP, a detection handle (typically either biotin or a fluorophore) must be attached. However, the relatively large size of the tag, compared with the size of the small-molecule the probe is modeling, could conceivably alter or reduce cellular interactions. Some of these reporter tags even render the probe less cell-permeant (Galmozzi et al. 2014), inhibiting the probe’s use in intact cells as opposed to cell lysates (Speers and Cravatt 2004). Bioorthogonal click chemistry, or more specifically copper-catalyzed azide-alkyne cycloaddition (CuAAC), can be utilized in order to attach the purification handle after cross-linking, making the probe more cell-permeant (Galmozzi et al. 2014). This is done by adding a terminal alkyne to the probe and an azide to the tag, or vice versa (Salisbury et al. 2007; Best 2009). ABPP with click chemistry has been used to study enzyme function in adipocytes (Galmozzi et al. 2014), targets of possible anticancer drugs (Wang et al. 2014; Speers and Cravatt 2004), as well the protein targets of oxidized lipids (Vila et al. 2008).
Beyond ABPP, photoaffinity probes can also be used to visualize sub-cellular localization through the use of fluorescence microscopy. Similar to the biotin handle, large fluorophores can alter a probe’s ability to cross the cell membrane and to localize in a manner similar to the drug of interest. Replacing the tag with a small CuAAC handle allows for the probe to diffuse into the cell and crosslink to the target. Following a fixation method, the probe can then be tagged with a complementary fluorophore and visualized. CuAAC has been used to localize platinum-based anticancer drugs (Qiao et al. 2014), to visualize protein synthesis inhibition by an analog of puromycin (Liu et al. 2012), and to label lipids (Neef and Schultz 2009). The Bertozzi group has done extensive cell-surface glycan labeling utilizing azide-derivatives of sugars and a strain-promoted copper-free version of CuAAC (Dehnert et al. 2012; Baskin et al. 2010; Laughlin et al. 2008; Baskin et al. 2007).

The CuAAC reaction is tolerant of a wide range of conditions, including aqueous environments, as well as various temperatures, redox conditions, and pHs (Himo et al. 2005). Since copper(I) is not stable in air, a copper(II) source is typically used, along with a reductant like sodium ascorbate, to produce copper(I) in situ (Hong et al. 2009). Toxicity of copper(II), as well as the production of reactive oxygen species by ascorbate, result in an inability to use CuAAC in live cells (Salisbury et al. 2007; Best 2009; Hong et al. 2009). Copper-chelating ligands have been designed to stabilize the active copper(I) in solution that have been optimized to reduce toxicity (Hong et al. 2009), or increase solubility of the ligand in aqueous solutions (Gupta et al. 2005). However, if CuAAC is used with purified protein, or following cellular fixation or lysis, the toxicity of copper(I) is not a concern. Furthermore, for photo-activatable probes, the probe itself is not amenable for studies of dynamic labeling, since activation results in covalent cross-linking and the inability to remove the probe from the target.
Taking all of the above into consideration, a new ABPP probe was designed, in order to incorporate CuAAC to attach the detection tag after photo-cross linking. Synthesis of a terminal alkyne probe of CQ was modeled after the synthesis of AzBCQ (Lekostaj et al. 2008). Instead of coupling a biotin on to the free terminal amine, a terminal alkyne was coupled onto the probe, yielding alkynylated azido chloroquine (AAzCQ), as shown in Scheme 4.1. The ability of AAzCQ probe to bind to PfCRT was then investigated through photolabeling of heterologously-expressed PfCRT purified and reconstituted into proteoliposomes photolabeling was compared to AzBCQ photolabeling. Subcellular localization of approximate cytoidal and cytostatic doses of CQ was investigated through the use of AAzCQ along with a commercially-available azide-derived fluorophore. Proteomics analyses, utilizing 2 dimensional gel electrophoresis with mass spectrometry, as well as avidin-enrichment with SDS-PAGE and mass spectrometry, was undertaken with the original AzBCQ probe, as well as AAzCQ, in order to identify cellular targets of chloroquine.
Scheme 4.1. Synthesis of AAzCQ. The synthetic pathway of AAzCQ is shown below. For step-by-step details, see Chapter II- Materials and Methods.

4.2 Results

In order to synthesize a novel ABPP probe that would integrate bioorthogonal coupling to provide a two-step system with added experimental versatility, the synthetic pathway previously used to synthesize AzBCQ (Lekostaj et al. 2008) was adapted to incorporate amide coupling of a terminal alkyne-containing carboxylic acid before esterification with the aryl azide for photoaffinity labeling, as shown in Scheme 4.1. Although the yield of the final reaction step is
quite low (less than 1%), milligram quantities were able to be made in high purity, allowing for moving forward into biological applications of AAzCQ.

Confirmation of product identity was obtained through examination of its $^1$H and $^{13}$C NMR spectra (Figures 4.4 and 4.5, respectively) and assignation of these peaks. For a detailed description of these peaks, see Chapter II- Materials and Methods. The final purification step required removal of a perfluoroazidophenyl-dimethylaminopyridine adduct from AAzCQ. Since the impurity contains fluorines, $^{19}$F NMR was used to determine final success of the purification and the percent purity of the final product. AAzCQ was successfully purified from the intermediate in a 95% purity, as shown in Figure 4.3.
Figure 4.5. $^{13}$C NMR of AAzCQ
Following successful synthesis of the proposed system, we needed to confirm that the new probe will crosslink under UV illumination to a biomolecule known to bind both CQ and the earlier probe AzBCQ. Proteoliposomes containing purified PfCRT (Dd2 isoform) from heterologous expression in S. cerevisiae yeast, which are known to bind both CQ (Zhang et al. 2004; Paguio et al. 2009) and AzBCQ (Lekostaj et al. 2008), were labeled successfully with AAzCQ. The labeling, shown in Figure 4.7, is concentration-dependent, as quantified through densitometry using Image J software (Rasband. Image J). This proof of principle experiment both confirms that AAzCQ can covalently bind to targets, and that the copper-catalyzed azide-
Figure 4.7. AAzCQ Labeling of PfCRT is Concentration Dependent. PfCRT (Dd2 isoform) in proteoliposomes (0.1 nmol protein) was labeled by 100 µM AzBCQ as a positive control (A. lane 2), as well as various concentrations of AAzCQ (100-500 µM). Coupled to 150 µM biotin azide after photolabeling, AAzCQ shows an increase in labeling with an increase in the initial probe concentration, (A. plot). UV illumination times were varied from 10 min (A. lanes 3-6) to 20 min (A. lane 7) and 40 min (A. lane 8) to ensure that the probe would crosslink. 10 minutes was long enough to see a signal on the blot. Blots were vs. biotin (A) and hexahistidine (B) as a loading control. Plot shown is the concentration of AAzCQ vs. the pixel density ratio (his:biotin) generated by Image J. software (Rasband. Image J.).
alkyne cycloaddition reaction that will be used to attach the visualization/purification handle to
the probe following crosslinking will proceed under biologically-relevant conditions, as the
probe was successfully crosslinked to azide-labeled biotin and visualized via streptavidin
Western blot. Additional UV-illumination time beyond the 10 min used for AzBCQ was
determined to not be needed.

In order to begin probing the *P. falciparum* parasite for additional targets at cytostatic and
cytocidal levels of CQ, a method for fluorescence microscopy was developed, as shown in
Scheme 4.2. This method crosslinks the ABPP probe within live parasites, and is then followed
by fixation and labeling with a fluorophore. For AzBCQ, the fluorophore used was a strepavidin-
linked Alexa Fluor 488 that in theory should bind to the biotin group on AzBCQ; for AAzCQ,
the fluorophore used was an azide-labeled Alexa Fluor 488 that should attach to the crosslinked
AAzCQ via CuAAC. Utilizing the same fluorophore core allows for better comparison between
the two systems, as the two probes will have identical spectral properties. Initial experiments
(data not shown) utilizing AAzCQ resulted in a high level of background labeling by azide-
linked fluorophores within the parasite cytoplasm during the CuAAC. I found that this labeling
did not depend on UV illumination, fixation procedures, permeabilization of the membranes,
presence or absence of the CuAAC catalysts, or the fluorophore core structure. In order to
prevent this non-copper catalyzed labeling from interfering with the examination of AAzCQ’s
targets, an azide-labeled tetramethylrhodamine (TAMRA) was incubated with samples in the
absence of the CuAAC-catalyst prior to fluorophore labeling of AAzCQ.

Examination of AzBCQ labeling at 2xLD$_{50}$ concentrations in both HB3 (CQS) and Dd2
(CQR) parasites shows clear fluorescence, (Figure 4.8). This labeling, however, seems to
include labeling within the RBC cytosol. It is possible that the streptavidin fluorophore’s large
size prevents complete access of the probe-labeled targets even after membranes are permeabilized, leading to non-specific staining. The high (μM) concentrations of probe required for cytocidal labeling result in the need for high concentrations of fluorescent probe. This method is compromised by the cost of obtaining the fluorophore-labeled antibody, as well as additional steps to fully reduce the formaldehyde used to fix the cells in order to avoid cross-reaction with the avidin protein. Overall, AzBCQ was determined to be a less than optimal probe for subcellular-localization experiments.
Scheme 4.2. Protocol for Fluorescence Imaging of Probe-Labeled Parasites. Parasites were labeled with AAzCQ by UV irradiation, crosslinking it to the target. The cells were fixed and the fluorophore was coupled through CuAAC. Mounting the cells to glass slides allowed for visualization by confocal microscopy.
Figure 4.8. Photolabeling *P. falciparum* with AzBCQ for Fluorescence Microscopy.

Trophozoite-stage parasites (HB3 and Dd2), within RBCS, were incubated with twice the LD$_{50}$ concentration of chloroquine of AzBCQ (250 nM for HB3 and 32,000 nM for Dd2) for 45 minutes followed by UV cross-linking for 10 min. Alexa Fluor 488-linked streptavidin was used to image with a spinning-disk confocal microscope. Scale bar is 10 µm.

Utilizing AAzCQ, HB3 and Dd2 parasites, in the trophozoite stage, were labeled at both their IC$_{50}$ and LD$_{50}$ concentrations, with 1x and 10x CQ competition to look for CQ-specific staining. As shown in Figures 4.9 and 4.10, both strains show AAzCQ-specific labeling (AF488 fluorescence) in small localized areas within the parasite in close proximity to the Hz-containing DV. The Hz can be visualized as the area in the parasite-blocking fluorescence (TAMRA) image as the dark area.
Figure 4.9. Photolabeling HB3 *P. falciparum* with IC$_{50}$ Concentrations of AAzCQ for Fluorescence Microscopy. Trophozoite-stage parasites (HB3) were incubated with IC$_{50}$ concentrations of chloroquine of AzBCQ (50 nM) with 0, 1 and 10 fold CQ concentrations. Alexa Fluor 488-azide was used to label the probe. TAMRA-azide was used to label the parasite. Images were obtained using a spinning-disk confocal microscope. Scale bar is 5 µm.
Figure 4.10. Photolabeling Dd2 *P. falciparum* with IC$_{50}$ Concentrations of AAzCQ for Fluorescence Microscopy. Trophozoite-stage parasites (Dd2) were incubated with IC$_{50}$ concentrations of chloroquine of AzBCQ (200 nM) with 0, 1 and 10 fold CQ concentrations. Alexa Fluor 488-azide was used to label the probe. TAMRA-azide was used to label the parasite. Images were obtained using a spinning-disk confocal microscope. Scale bar is 5 µm.
Figure 4.11. Photolabeling HB3 *P. falciparum* with LD$_{50}$ Concentrations of AAzCQ for Fluorescence Microscopy. Trophozoite-stage parasites (HB3) were incubated with LD$_{50}$ concentrations of chloroquine of AzBCQ (150 nM) with 0, 1 and 10 fold CQ concentrations. Alexa Fluor 488-azide was used to label the probe. TAMRA-azide was used to label the parasite. Images were obtained using a spinning-disk confocal microscope. Scale bar is 5 µm.
Figure 4.12. Photolabeling Dd2 *P. falciparum* with LD$_{50}$ Concentrations of AAzCQ for Fluorescence Microscopy. Trophozoite-stage parasites (HB3) were incubated with LD$_{50}$ concentrations of chloroquine of AzBCQ (15 µM) with 0, 1 and 10 fold CQ concentrations. Alexa Fluor 488-azide was used to label the probe. TAMRA-azide was used to label the parasite. Images were obtained using a spinning-disk confocal microscope. Scale bar is 5 µm.
surrounded by fluorescence. In both strains, this IC$_{50}$ labeling is competed by the underivatized CQ, resulting in little-to-no fluorescence of AAzCQ in the samples containing 10x CQ.

Under LD$_{50}$ conditions, both strains show more fluorescence intensity spread throughout the entirety of the parasite cytoplasm, as shown in Figures 4.11 and 4.12. Like the cytostatic concentration labeling, this cytocidal labeling is also competed away by the presence of underivatized CQ, particularly at a ten-fold excess. The difference in localization of the AAzCQ-specific fluorescence is not due to concentration alone. The IC$_{50}$ concentration used for Dd2, 200 nM, Figure 4.7, is actually higher than the LD$_{50}$ concentration used for HB3, 150 nM, Figure 4.8. If the labeling redistribution were purely from the addition of more probe, we would not expect to see the lower amount of labeling seen in IC$_{50}$ Dd2 labeling, which is limited in its spatial distribution compared to the HB3 LD$_{50}$.

The fluorescence microscopy shows that the AAzCQ probe can label different spatial regions of the parasite at cytocidal concentrations as compared to cytostatic. In order to try to identify what the targets might be that are labeled at cytocidal concentrations, bulk quantities of parasites can be labeled for proteomic analysis. Scheme 4.3 shows one method of analyzing whole-cell parasite lysates for labeled proteins. Following covalent crosslinking of the probe to its target, the cells can be lysed, and AAzCQ conjugated to biotin. If AzBCQ is used, the conjugation step is not needed. Two-dimensional gel electrophoresis can then be used to separate the proteins; with Western blotting against biotin used to identify what spots could be conjugated to the probe. A companion gel that has been stained with Coomassie Blue can then be used for spot excision and trypsin digestion of the protein for mass spectroscopic analysis.
Scheme 4.3. Protocol for Separation of Biotin-Labeled Targets with 2D Gel Electrophoresis.

Parasites are incubated with, and crosslinked to the probe with UV light. Conjugation to biotin allows for visualization of protein targets via Western blot after 2D gel electrophoresis. Excision of labeled spots allows for MS analysis of trypsin-digested peptides and protein identification.
Figure 4.13. Two-Dimensional Gel Electrophoresis of Bulk *P. falciparum* Labeled with AzBCQ. Bulk cultures of HB3 (A and B) and Dd2 (C and D) *P. falciparum* were labeled with 10 µM AzBCQ, and run on two 2D Gels for Western blot with streptavidin-HRP (A and C) or coomassie staining for excision, trypsin digestion, and mass spectroscopic analysis (B and D). Grids were superimposed over the blots and gels for aid in comparison and excision of proteins from the Coomassie gel for MS analysis.

This method was used as an initial attempt at identifying possible probe-labeled targets with AzBCQ. The Western blots and Coomassie gels are shown in Figure 4.10. Aligning a grid over the blots and gels allows for comparison between the spots detected on the Western Blot
and the proteins present on the Coomassie gel. Note that biotin imaging (Figure 4.13 A, C) is much more sensitive than Coomassie Blue staining, however equal amounts of protein are run on A and B, as well as C and D. Since Coomassie Blue will stain any protein, the Western Blot is needed to specify where in the gel the spots of interest are. Both strains show spots labeled with biotin in the region of C4-C5/D4-D5. The intensity of the spots varies between the two strains, with HB3 labeling the C4 spot much more intensely than Dd2, with the opposite true for the C5 spot.

**Table 4.1. Plasmodium Proteins Identified Through Trypsin-Digestion and Mass Spectroscopic Analysis of Bulk P. falciparum Labeled with AzBCQ. Possible hits are in bold.**

<table>
<thead>
<tr>
<th>Spot Location</th>
<th>Plasmodium Protein Identified</th>
<th>Number of Matching Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB3 C/D 4/5</td>
<td>Proteasome subunit alpha type</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Triosephosphate isomerase</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Heat shock 70 kDa protein</td>
<td>2</td>
</tr>
<tr>
<td>HB3 D 1</td>
<td>Putative protein kinase</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Qa-SNARE protein</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Serine repeat antigen 6</td>
<td>1</td>
</tr>
<tr>
<td>HB3 C2</td>
<td>Uncharacterized protein Q8IBZ1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Putative Rrp6 homologue</td>
<td>1</td>
</tr>
<tr>
<td>Dd2 C/D 5</td>
<td>Uncharacterized protein Q8I659</td>
<td>1</td>
</tr>
<tr>
<td>Dd2 C2</td>
<td><strong>Endoplasmic reticulum-resident calcium binding protein</strong></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Actin-1</td>
<td>8</td>
</tr>
<tr>
<td>Dd2 C3</td>
<td>Actin-2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Putative uncharacterized protein Q8ILN7</td>
<td>1</td>
</tr>
<tr>
<td>Dd2 D1</td>
<td>Uncharacterized protein Q8IBZ1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Uncharacterized protein Q8I659</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Putative Dynein heavy chain</td>
<td>1</td>
</tr>
</tbody>
</table>
The D4/5 spot in HB3 is possibly shifted to a higher pI value in the Dd2 gel, as the spot resides within D5 alone. The same possible shift is observed in the C2 spot in HB3, which resides on the border of C2 and C3 in Dd2. D1 also shows variation between the two strains, as the spot in HB3 is much less intense than Dd2, with Dd2 having an additional spot on the border with D2.

Mass spectroscopy of trypsin-digested peptides was used to investigate what proteins may be present in these spots of interest. The identified proteins are listed in Table 4.1. Some possible identifications were made through only 1 peptide, making these assignations not very strong. Proteins identified with more than 1 peptide include a component of the proteosome, triosephosphate isomerase and a 70 kDa heat shock protein in HB3, and an a calcium-binding protein from the endoplasmic reticulum and two types of actin in Dd2. Further examination of the peptide masses shows no peptide masses that could also include the added 765.25 amu of AzBCQ above the lower-limit cut-off of the system, (no peptides below 400 Da are detected). Without confirmation of AzBCQ binding to a measured peptide, it is not possible to unequivocally prove what proteins in the excised region were actually crosslinked to the probe.

A second method of protein isolation and separation was developed that utilizes avidin-enrichment of biotin-containing proteins, as shown in Scheme 4.4. In order to optimize the protocol and choose between monomeric avidin (lower affinity) or streptavidin (higher affinity) resin for enrichment a pilot “proof of principle” experiment was performed. In brief, a biotinylated PfCRT from crude membranes of *S. cerevisiae* were enriched and run on one-dimensional SDS-PAGE gels for visualization of biotin-containing proteins with Western blot and a visualization of total protein with a silver stain, as shown in Figure 4.11. Monomeric
avidin enrichment, shown in Figure 4.14 A and B, shows biotin-containing proteins in the first washes (lanes 3 and 8), and not in the elutions (lanes 5 and 10), suggesting that the biotin-avidin interaction in these beads is not strong enough to enrich an elution with biotinylated proteins. Streptavidin enrichment, shown in Figure 4.14 C and D shows less biotinylated protein in the first wash, particularly for 2 µg of initial protein (lane 3). The fourth wash for both 2 and 10 µg of initial protein shows no biotinylated protein. In both samples, elution of the avidin-captured proteins by boiling in Laemmli buffer (lanes 5 and 10) yields biotinylated proteins with little contaminating protein seen in the silver stain. The higher affinity of streptavidin is preferable to the lower affinity of monomeric avidin, especially since boiling samples in Laemmli buffer before gel electrophoresis is acceptable for downstream mass spectroscopy analysis.
**Scheme 4.4.** Method for Avidin Enrichment of Biotin-Labeled Targets. Parasites are incubated with, and crosslinked to the probe with UV light. Conjugation to biotin allows for enrichment of protein targets on streptavidin beads, followed by elution and visualization via Western blot after 1D gel electrophoresis. Excision of labeled bands allows for MS analysis of trypsin-digested peptides and protein identification.
Figure 4.14. Avidin Enrichment of Biotin Acceptor Domain-Linked PfCRT from Yeast Crude Membranes. Monomeric avidin (A and B) and Streptavidin (C and D) were used to isolate PfCRT tagged with a biotin acceptor domain. Biotin blots (A and C) and silver stained gel (B and D) are shown. Red arrow denotes PfCRT. Lane 1. Ladder Lane 2. 2 µg starting crude membrane protein Lane 3. Wash 1 Lane 4. Wash 4 Lane 5. Elution Lane 6. Empty Lane 7. 10 µg starting crude membrane protein Lane 8. Wash 1 Lane 9. Wash 4 Lane 10. Elution.

Following the protocol in Scheme 4.4, and using the streptavidin beads for enrichment, cell lysates of AAzCQ-labeled parasites were coupled by CuAAC to biotin azide. AzBCQ-
labeled parasites were lysed and continued alongside the coupled AAzCQ samples. Following precipitation of proteins to remove excess biotin, the resolubilized proteins were incubated with streptavidin-linked beads, where proteins without a biotin moiety were washed away. Separation of the eluted biotin-containing proteins was then performed by one-dimensional SDS-PAGE for excision of bands of interest. CQ-competition (1,000x) was also utilized to look for bands with CQ-specific labeling.

An advantage to this method is that unlike two-dimensional gel electrophoresis, where each individual sample must be run on its own gel, all samples for each strain can be run on the same gel with avidin-enrichment. This allows for more direct comparison between the two probes as well as labeling in the presence vs. absence of CQ-competition. Figure 4.15 shows a biotin Western blot for HB3 parasites labeled with both probes. An intense band is observed in every sample, both the washes and the elutions, which is denoted in the cartoon by the red line. In the AzBCQ-labeled elutions (lanes 3 and 6), below this band are two less-intense bands that are present but reduced upon CQ competition, denoted by the purple and green bands. The green band is also present in the AAzCQ-labeled elution (lane 9) alongside a band above the red line (blue) and both are competed away by CQ (elution in lane 12). These bands of interest, unlike the red band, are not very intense and are probably present in amounts reaching the lower limit of detection in biotin Western blotting thus they are not easily visible by Coomassie staining. The Coomassie Blue-stained gel only shows clear bands in the non-enriched sample and the washes. Nonetheless, excision of the areas of interest in the elution lanes are directed by the biotin blot, and corresponding regions from the Coomassie gel using grids (similar to those shown in Figure 4.13) are then excised for MS analysis.
Figure 4.1. One-Dimensional Gel Electrophoresis of Bulk *P. falciparum* Labeled with AAzCQ and AzBCQ with CQ Competition in HB3 Parasites. Top left is the biotin blot, which is recreated in cartoon form on the top right. Lower left is the companion Coomassie Blue-stained gel used for mass spectroscopic analysis.

Figure 4.16 shows the biotin Western blot for Dd2 parasites labeled with both probes. The same intense band through all samples also appears in the Dd2 gel, and is again denoted by the red line. In the AzBCQ-labeled elutions (lanes 3 and 6), the purple and green bands appear again, with a reduction in labeling in the presence of CQ competition. Neither of these bands is present in the AAzCQ-labeled elution (lane 9). An additional band, intensely labeled at a lower
Figure 4.16. One-Dimensional Gel Electrophoresis of Bulk *P. falciparum* Labeled with AAzCQ and AzBCQ with CQ Competition in Dd2 Parasites. Top left is the biotin blot, which is recreated in cartoon form on the top right. Lower left is the companion Coomassie Blue-stained gel used for mass spectroscopic analysis.

Mass value, marked by the orange bar, occurs in the AzBCQ-labeled sample and is competed by CQ. Like the HB3 gels, these bands of interest are not very intense yet excision of the areas of interest in the elution lanes will be directed by the biotin blot. Proteomic analysis of these provocative data is ongoing. In the future, similar experiments will be done with other CQS and CQR strains.
4.3. Discussion

AAzCQ, an ABPP probe with increased experimental flexibility compared to AzBCQ was synthesized and utilized to probe for cytotoxic targets of CQ in *P. falciparum* parasites. This crosslinking interaction is extremely fast. Singlet nitrenes have lifetimes in the femtosecond lifetimes (Burdinski *et al.* 2006), so if the target is weakly bound, it is likely that the nitrene will decay through a side reaction and not insert into a neighboring C-H single bond. Utilizing CQ competition helps to identify specific targets, but must be present before UV-crosslinking due to the short lifetime of the nitrene.

I find that accurately analyzing subcellular distribution of ABPP probes of CQ strongly benefits from the use of AAzCQ. Initial experiments utilizing AzBCQ with a streptavidin-linked fluorophore showed additional fluorescence within the cytoplasm of the RBC, not within the parasite, even with permeabilization of the membranes. It is possible that the large size of the streptavidin compared to that of small molecules like AzBCQ prevents it from labeling specifically. Nonspecific protein-protein interactions could be the cause of the high background staining observed, particularly since cell are fixed with paraformaldehyde (a protein crosslinker) and mounted to coverslips through the electrostatic interaction of the phosphate head groups of the membrane with polylysine.

AAzCQ, since it utilizes a small-molecule fluorophore, does not seem to suffer from the same shortcomings. Early experiments, however, yielded a different problem with background staining. Despite the bioorthogonal-nature of aliphatic azides, there was non-probe specific staining of the parasite cytoplasm by azide-linked fluorophores. This staining was not determined to be due to UV illumination, fixation procedures, permeabilization of the
membranes, presence or absence of the CuAAC catalysts, or the fluorophore core structure, data not shown. In order to reduce the non-probe specific labeling, “blocking” was adopted. Following probe crosslinking and fixation/permeabilization of the cells, the samples were incubated in the presence of TAMRA-azide in the absence of the catalysts, and then washed. Incubation in Alexa Fluor 488-azide with the catalysts afterwards yielded probe-specific labeling. The TAMRA-azide could be used to visualize the parasite cytoplasm, with the areas of no fluorescence corresponding to the hemozoin-containing DV.

Imaging HB3 and Dd2 parasites at their IC_{50} levels with AAzCQ yields labeling that is limited in its spatial distribution through the parasite cytosol, and is typically within close proximity of the low-TAMRA fluorescence regions containing Hz. Excess CQ concentrations successfully reduce this labeling, suggesting that the labeled targets’ binding sites overlap with those of CQ. The distribution of LD_{50} concentrations of the probe, as well as the intensity, is increased in both strains, even though the LD_{50} concentration for HB3 is actually lower than the IC_{50} concentration for Dd2. This suggests that the increased labeling under cytocidal conditions is not a simple increase in signal resulting from an increase in absolute concentration, but due to an alteration of the localization of the targets. It is possible that under cytocidal conditions, the probe is labeling new protein targets that are not labeled under cytostatic conditions, and that these targets are spread throughout the cytosol. This labeling is CQ-competed as well, suggesting that the targets are CQ-specific. This data supports the hypothesis that cytostatic and cytocidal targets of CQ in *P. falciparum* are distinct. This is consistent with other studies published recently in our lab that cytocidal doses target more than just Hz. These observations include: a redistribution of ATG8 following cytocidal CQ treatment (Gaviria *et al.* 2013), activity of high
CQ doses against non-Hz forming stages, and an alteration of the number of daughter nuclei and viability of the following cell cycle (Gligorijevic et al. 2008).

The subcellular localization of AAzCQ can suggest distinct targets, but proteomic analysis is needed to determine what those targets could be. Initial exploration of possible cytocidal CQ targets was performed with AzBCQ, with my logic being that the signal would not be reduced by adding the CuAAC reaction step that could conceivably lower overall yield. Separation of cellular lysates was performed by two-dimensional gel electrophoresis. Western blots detected biotin-labeled proteins in both strains, but comparison of the distribution of these spots is made difficult by the lower reproducibility of 2D gels. Since each sample has to be run on its own gel, samples cannot be run side-by-side with standards. And although the individual gels can be run side-by-side, this technique is more susceptible to slight variations in absorbing protein lysates into the isoelectric focusing strip, and mounting the focusing strip into the SDS-PAGE gel for mass separation. Additionally, few proteins were detected on the Coomassie stained gel, making excision more difficult. This may have been due to lower amounts of protein that could be absorbed by the focusing strip due to incomplete solubilization of the lysate in the nonionic detergent required for 2D gel electrophoresis.

Nonetheless, the mass spectrometric analysis of the 2D gels provides some possible proteins in the regions of interest that include, in the HB3 sample, a proteosome subunit and heat shock protein 70. Proteosomes are an integral part of the ubiquitin-dependent protein degradation pathway and are localized to the cytoplasm and the nucleus. The alpha subunits, of which the identified protein would be a part, act as a substrate gate, allowing substrates access to the beta subunit for degradation (Sridhar et al. 2013). The localization of the proteosome to the cytoplasm
is consistent with the subcellular localization observed with AAzCQ, as well as the hypothesis that cellular survival pathways that rely on degradation of damaged biomolecules may be involved in the action of antimalarial compounds. Heat shock protein 70, and ATP-dependent cellular chaperone involved in maintaining proper protein folding, is found in all cellular compartments, including the cytosol, parasitophorous vacuole, and Maurer’s clefts (Shonhai et al. 2007). An additional protein hit was triose phosphate isomerase, which has been localized to the Maurer’s clefts and PVM in Plasmodia (Aurrecoechea et al. 2009). Isoforms of that protein have localized to both the apicoplast and the cytosol (depending on the isoform) in the fellow Apicomplexan parasite Toxoplasma gondii (Fleige et al. 2007).

The Dd2 gel MS analysis did not include any stress-response proteins, with the only proteins identified through more than 1 matching peptide including a calcium-binding protein from the endoplasmic reticulum, as well as the structural protein actin. Endoplasmic reticulum-resident calcium-binding protein localizes, as expected, localizes to the endoplasmic reticulum. The protein has been hypothesized to be involved in protein trafficking (La Greca et al. 1997), and has been identified as a target of synthetic endoperoxides N-89 and N-251 (Mortia et al. 2012). The Plasmodium falciparum genome encodes 2 isoforms of actin, both of which were identified in the two-dimensional gel electrophoresis MS analysis. Actin I has been determined to have functions in several processes, including host cell invasion, cell motility, and vesicle trafficking. Interestingly, it has been hypothesized to be involved in host Hb uptake (Deligianni et al. 2011). Hoppe et al. (2004) have observed that CQ treatment results in an increase in Hb-containing vesicles in the parasite possibly due to the inability to be properly trafficked to the DV for proper fusion. Actin II was also identified in the MS analysis. Gene deletion experiments
have determined that actin II is not necessary for asexual-stage viability, but is involved in generation of male gametocytes, and is necessary for generation of their flagella (Deligianni et al. 2011).

Although differential labeling in CQS vs. CQR parasites is expected, in order to identify targets present in the CQS strain that are not in the CQR, the low peptide matching, as well as the inability to assign any peptides as having AzBCQ crosslinked to it, suggests that this technique may not be the best method of proteomic analysis of CQ targets.

A second protocol was designed based on biotin-avidin affinity, and optimized through the use of crude membranes of yeast containing a biotinylated PfCRT. Enriching cell lysates for biotin-containing proteins allows for one-dimensional separation through SDS-PAGE, and the side-by-side running of multiple samples with mass standards. Analysis of HB3 and Dd2 parasites labeled with both probes with CQ competition shows both differential labeling between the strains and between the two probes. The set of green bands, of a mass around 50 kDa, are labeled, and competed with CQ, by both probes in HB3, with the competition of AAzCQ reducing labeling below the ability to detect it. The purple band is labeled by AzBCQ in HB3 with CQ competition reducing labeling. These bands are also labeled by AzBCQ, and competed with CQ, in Dd2, but not AAzCQ. HB3 labeled by AAzCQ shows an additional band above 60 kDa that is competed with CQ; while Dd2 shows an intense band in AzBCQ at about 30 kDa that is competed with CQ, but not labeled with AAzCQ. MS analysis of these provocative bands is underway.

AAzCQ provides an important new tool in identifying possible targets of CQ cytocidal treatment in *P. falciparum* parasites. Examination of additional sensitive and resistance strains,
as well as comparison of identified proteins with genomic regions identified as being associated with cytocidal CQR (see Gaviria et al. 2013), can help to identify how parasites have evolved to resist CQ’s toxic effects, allowing for an understanding of the mechanisms of resistance utilized by this deadly pathogen.

4.4 Acknowledgements

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CHAPTER V
IDENTIFYING NOVEL DRUG COMBINATIONS AND DEFINING THEIR INTERACTIONS AT CYTOSTATIC AND CYTOCIDAL DOES

This work to be published as:

5.1 Background

The rise of resistance against many forms of malaria treatment, from chloroquine and fellow quinolines to the combination therapy of sulfadoxine-pyrimethamine, has lead to the search for new antimalarial drugs and a renewed focus on development of combination therapies. The WHO currently recommends drug combinations including: CQ, AQ, or MQ with sulfadoxine-pyrimethamine, atovaquone/proguanil, QN with either tetracycline or doxycycline, as well as artemisinin combination therapies (ACTs), which include the artemisinin (ART) derivatives artesunate (ATSU), artemether (ATM), and dihydroartemisinin (DHA). ACTs include: ATSU/CQ, ATSU/AQ, ATSU/sulfadoxine-pyramethamine, ASTU/MQ, and ATM/LF (Greyer, ed. 2001). Combinations of two or more drugs are advantageous because it should delay the rise of resistance due to the impact of multiple targets within the same pathway or spread between multiple pathways by requiring additional mutations to overcome the toxic insult
of both compounds. The parasite would have to evolve to alter both targets (or their pathways) as well as any additional negative impacts that could result from synergistic interaction of the partner drugs. The presence of partner drugs help to eliminate any mutations within the population that might result in resistance against either drug as a monotherapy (Hastings et al. 2002).

In formulating an ACT pairing, the ART derivative is typically used alongside a partner drug with a longer half-life, to ensure full clearance of the infection, as well as a different mechanism of action (MOA), if known. When ATM was partnered with the pseudo-quinoline lumefantrine (LF), preclinical studies reported synergistic interactions between the two drugs (Olliaro et al. 1995). Much discussion on ATM and LF as partner drugs focuses on their complementary pharmacokinetics. When ATM is administered, it is rapidly absorbed and partially metabolized to DHA, which both have a plasma half-life of about 1 hour. LF, which is absorbed much more slowly, is also eliminated slowly; its half-life is between 3 and 6 days. The use of a rapidly-absorbed, highly-active compound like ATM alongside a slowly-absorbed, slowly-cleared compound like LF is meant to ensure quick mitigation of the symptoms via a rapid drop in parasitemia (from $10^9$-$10^{12}$ to $10$-$10^4$ parasites) as well as full clearance over a longer period of time to prevent relapse (White et al. 1999). A fixed amount ratio of 1:6 ATM:LF, now used under the trade name “Coartem”, was observed to be the optimum combination to administer in early trials, and resulted in higher parasite clearance and a higher cure rate, when compared to monotherapy with either drug (White et al. 1999; Novartis Pharmaceuticals Corporation. Coartem Product Information). This pairing is also meant to help avoid the rise of resistance, due to differing hypothesized mechanisms of action between the
reactive endoperoxide of ATM that results in radical alkylation of target proteins and the pseudo-quinoline properties of LF, which may inhibit the growth of hemozoin.

The reports of *in vitro* synergy of ATM and LF typically trace back to a study by Alin and coworkers in 1999 that utilized a commonly-used technique known as checkerboard analysis to quantify the interaction via a probit method of data analysis. Examining two strains, T-996 and LS-21, the authors noted synergy at the IC50, IC90, and IC99 levels at 100:1, 30:1, 1:100 (ATM:LF) concentration ratios, by converting growth data into quantile units for probit analysis. Probit analysis is a weighted regression system that allows inclusion of all data from 0-100% as a linear trend, as opposed to the sigmoidal curve-fitting common in our laboratory (Tallarida 1992). The determination of synergy is based on predicted growth inhibition by two compounds acting independently (derivation not discussed) (Alin *et al.* 1999).

Additionally, a study in 2007 by Thriemer *et al.* identified synergy between LF and the DHA on a series of isolates from Bangladesh by utilizing the isobologram method. However, the use of isolates, rather than laboratory-adapted strains, makes it more difficult to generate growth data over a 72 hour period; antiplasmodial activity could only be generated for half of the 10 isolates collected (Thriemer *et al.* 2007). By using two quantitative methods (isobologram and combination index analyses) that rely on growth curve data, and that take into account the shape (provided it remains the same between monotherapy and combination therapy), this study provides reliable quantification, and clear assignation of the synergistic effect of DHA and LF within those 5 tested isolates.

In another examination of the impact of metabolism, Wong *et al.* identified slight synergy between DHA and the LF metabolite desbutyl-lumefantrine utilizing the same technique as the
Thriemer study. While not as clearly synergistic as the Thriemer results, the Wong study does utilize laboratory-adapted strains. This allows for repetition of the assays performed, and more statistically-rigorous results. The study also looked for possible interactions between LF and desbutyl-lumefantrine (designated additive), but did not examine LF and ATM, or LF and DHA. If these two combinations were studied, it would be possible to know if the trends observed by Alin and Thriemer extended to other, more commonly studied strains (3D7 and W2mef) (Wong et al. 2011).

Techniques to experimentally identify synergistic partner drugs *in vitro* include altering the concentrations and relative ratios of drugs through a checkerboard pattern on the assay plate, as well as fixed-ratio serial dilutions. Either technique can be used to generate the most commonly used plot of the interaction, known as an isobologram. Isobologram analysis requires comparison of the growth curve data of the drug in the presence of its partner with its monotherapy activity, generating a ratio of the activity in combination with the activity as a monotherapy, known as a fractional inhibitory concentration (FIC). FIC values are calculated for each compound at a series of fixed ratios between the two. When these data are plotted, the results can be a straight line between (1,0) and (0,1) (additive), a concave curve (synergistic), or a convex curve (antagonistic) (see Figure 5.1). More complex curves that are a mixture of these shapes are also possible, if the interaction depends on the ratio between the two compounds, meaning that some combinations may in fact be synergistic at certain ratios and additive or antagonistic at others (Berenbaum 1978).
Figure 5.1. Example of Possible Drug Interactions on an Isobologram. Hypothetical drugs A and B are examined to calculate their FIC values at 5 fixed ratios, and then plotted and the shape of the curve identified as it compares to the mathematical additive line (dotted) that connects (1,0) and (0,1).

Generating a full isobologram requires the analysis of various drug ratios, and results in a large number of required assays. Derived from the Law of Mass Action, a simpler and mathematically sound method for identifying drug interaction type was developed by Chou and Talalay, known as the Chou-Talalay Method (C-T) (Chou 2010). Due to its mathematical derivation from a fundamental law, this technique can be used for the examination of interactions.
in vitro and in vivo, is applicable for primary and secondary ligands, and is not dependent on the
mechanism of action of any tested compound. In any analysis of possible synergy, the
fundamental core of defining interactions is in defining additivity. Where other techniques can
only do so for mutually-exclusive (isobologram) or non-mutually-exclusive (fractional-product
method) compounds, the definition of additivity in the Chou-Talalay Method is tolerant of both
types on interaction (Chou et al. 1984).

In order to analyze a combination at a certain ratio of the test compounds by C-T, the
FICs are calculated in the same way as one would do for an isobologram. These values must be
calculated from a dose-response curve generated from multiple data points. Examination of a
single growth data point does not incorporate the shape of the curve into the quantification,
negating one of the advantages of C-T analysis. Once the FIC values are calculated, instead of
plotting them on a graph, these values are summed to generate a combination index, or fractional
inhibitory concentration index (FIC\textsubscript{index}). Based on their derivation, Chou and Talalay assigned
additivity as occurring when the FIC\textsubscript{index} equals 1. When the FIC\textsubscript{index} is less than 1, the interaction
is synergistic; when it is greater than 1, the interaction is antagonistic. This methodology can be
applied to a combination of any number of different compounds, although the experimental
requirements for data generation may limit the number of compounds in the combination (Chou
et al. 1984). Although the assigned cut-offs hold mathematically, experimental variance leads to
deviation from these values. The cut-offs used in experimental analysis can vary from synergy
less than 0.5 and antagonism greater than 4 (Bell 2005), to just expanding the additive range to
between 1 and 2 (Matthews et al. 2013). The choice of cut-offs can be somewhat arbitrary,
depending on experimental error and variability (Bell 2005).
Until recently, all examination of antimalarial drug combinations in vitro focused on cytostatic conditions, where the effects of low concentrations of drug on growth rates are examined. Recent work has shown that, in addition to these cytostatic effects, many antimalarial compounds have cytocidal (or parasite-kill) properties (Paguio et al. 2011; Gaviria et al. 2013). When compared to cytostatic effects, different patterns of drug susceptibility and resistance can be identified under cytocidal conditions (Paguio et al. 2011; Gorka et al. 2013a; Gorka et al. 2013d). When applying this distinction to combination analysis, it is possible that the interaction of two compounds may vary between low-dose growth inhibition and high-dose cell kill, both between susceptible and resistant strains, but also within the same strain under different dosing conditions. One study, by Gorka and coworkers in 2013, explored both of these conditions with common quinoline antimalarials using isobologram analysis. In some cases, the cytostatic combination could vary quite significantly between susceptible HB3 and resistant Dd2. For example, amodiaquine and tafenoquine are additive in HB3 but quite antagonistic in Dd2. Between low and high dose the interaction can vary quite significantly as well. The combination of CQ and amodiaquine in Dd2 parasites is additive at the cytostatic level, but highly antagonistic under cidal conditions. This combination analysis was able to identify synergy between tafenoquine and methylene blue under static and cidal conditions in Dd2, with HB3 synergistic at cidal, and additive at cytostatic doses. Differences like this reinforce the need to examine possible novel combination therapies at both levels to ensure additive or synergistic interaction in a malaria patient (Gorka et al. 2013b). Additive interactions do not preclude the use of a certain combination, particularly if higher, cidal doses can result in an increase in synergy.
Circumventing the drug resistance currently observed in clinical isolates and laboratory strains, and slowing the advent of future resistance, requires the identification of novel drug combinations (Lukens et al. 2014). By clearly defining synergistic, additive, and antagonistic effects \textit{in vitro}, it is possible to identify drug pairings that exert maximal antiplasmodial activity at minimal doses, possibly reducing negative off-target effects in the host. However, it is necessary to explore these interactions at both the cytostatic and cytocidal level. Just because synergy or additivity is observed at one dose does not mean the same is seen at the other. As the concentration of a drug is taken up by the host, it can reach cytocidal levels, and then approach cytostatic as the drug clears the system; potential combination therapies should be examined under both sets of conditions to avoid antagonistic pairings that will reduce treatment efficacy.

Combinations of the selected PI3K inhibitors and currently-used antimalarials were analyzed for the nature of their interaction \textit{in vitro}, as well as their impact on cellular autophagy with the goal of identifying novel combinations that are active against sensitive and multidrug-resistant lines of \textit{Plasmodium falciparum}.

\textbf{5.2 Results}

High throughput screening of a library of antimalarials, FDA approved drugs, and compounds under clinical review at the National Center for Advancing Translational Science (NCATS) was performed by our collaborators on 4650 combinations to date. A comprehensive set of 240 combinations of interest were then screened in duplicate against two individual cultures for each of the three parasite strains (3D7, HB3 and Dd2) as shown in Figure 5.2A (Mott et al. 2014, in preparation). After removing assays that failed to meet QC criteria (see Materials
and Methods), several metrics were applied to determine the combination response in order to prioritize those that could be explored as potential antimalarial therapies, including hierarchical clustering and mechanism of action relationships. Some compounds were selected for closer analysis as partner drugs to currently-used antimalarials artemether and lumefantrine. Shown in Scheme 5.1, these compounds included GSK2126458 (GSK212), NVP BGT226 (NVP), Torin 2, and PIK 93. All four of these compounds are known to be inhibitors of phosphotidylinositol 3-kinases (PI3Ks). PI3Ks are a large family of enzymes, but include enzymes involved in the autophagy cascade. Genomic analysis has yet to identify an mTOR (class IV PI3K) homolog in *Plasmodia* (Hanson *et al.* 2013), but a homolog of the class I PI3K VPS34 has been identified (Gaviria *et al.* 2013).
Figure 5.2. Single agent and combination analysis of a large collection of approved and investigational drugs for antimalarial activity. (A) Heat map representation of approved and investigational drugs (data available at http://pubchem.ncbi.nlm.nih.gov/). (B) Combination data (10×10 plots) for drug combinations representing the standard of care (ATM + LF, ATSU + MQ) and for novel combinations (MQ + nicardipine (NIC), ATM + Alvespimycin, Atovaquone + ML238, Amodiaquin + BIX-01294, Methylene Blue + Tafenoquine. ATM + NVP-BGT226). Growth data from the heat map presentation can be processed for identification of synergy through generation of an isobologram, or calculation of deviations from the Bliss Model (see Chapter II- Materials and Methods, section 2.2.12 High-Throughput Screening of Drug Combinations).
Scheme 5.1. Structures of tested PI3K inhibitors, as well as antimalarials artemether and lumefantrine.

The approved and investigational drugs included in each matrix screen included a collection of known antimalarials such as DHA, ATM, ATSU, CQ, mefloquine (MQ), AQ and piperaquine (PPQ), as well as drugs used in many other diseases. As the current frontline of malaria therapy, ACTs, including ATM-LF, AS-MQ, ATSU-pyronaridine, ATSU- AQ, DHA-PPQ, were also analyzed. Consistent with previous reports both ATM-LF and ATSU-MQ were noted to interact favorably (Fig. 5.2B) (Cokol et al., 2011; Alin et al. 1999). In addition to currently used ACTs, many novel combinations were determined to by synergistic or additive.
These included combinations of currently approved antimalarial drugs with compounds such as ion channel modulators (e.g. nicardipine), novel mitochondrial targeting agents (e.g. ML238), drugs targeting human enzymes and receptors (e.g. BIX-01294, alvespimycin and NVP-BGT226), as well as agents currently undergoing single agent clinical assessment in malaria trials (e.g. tafenoquine) (Fig. 5.2B). Further examination of these combinations yielded certain classes of compounds that trended towards favorable interactions, including compounds targeting the mitochondrion, ion channel blockers, HSP90 and PI3K targeting drugs, and known antimalarials were noted.

One class of compounds identified in the high throughput screen were drugs that modify ion homeostasis in combination with the ART class. Ion homeostasis plays a significant role in parasite biology and the interaction of ion channel blockers and antimalarial drugs had previously been documented (Garcia 1999). Ion channel antagonists such as nicardipine (shown in Fig 5.2B), manidipine, manoalide (Ca$^{2+}$) and propafenone (Na$^{+}$) (data not shown) were synergistic with the ARTs and quinolines (Weisman et al. 2006). Other modulators of ion homeostasis such as the small molecule KN-62 demonstrated strong synergy with many approved antimalarial drugs (Fig 5.2B). This agent is an inhibitor of the human Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII), which previously has been shown to perturb cytosolic Ca$^{2+}$ flux and disrupt or modulate cell signaling pathways in *Plasmodium* (Mahata et al. 1996). Single cell photometry was used to study calcium homeostasis for parasites treated with these drugs, and some drug combinations. My colleagues Dr. David Gaviria and Amila Siriwardana observed an acute release of Ca$^{2+}$ from the parasites digestive vacuole (DV).
upon treatment with CQ with a corresponding increase of cytosolic Ca\(^{2+}\) (see Figure 5.3, Gaviria, D.; Siriwardana, A.; Roepe, P.

**Figure 5.3.** Calcium Loss Under Cytocidal Chloroquine in Dd2 Parasites. Time lapsed capture of DV localized Fura 2 showing rapid loss of DV Ca\(^{2+}\) for CQR Dd2 parasites under perfusion upon introduction of cytocidal doses of CQ.

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In order to further probe the possible synergies of ATM and LF with the selected PI3K inhibitors, compounds were screened for their IC\(_{50}\) and LD\(_{50}\) activities, as shown in Table 5.1. NVP and Torin2 were both identified as highly potent at the cytostatic level (low nM), while NVP is also highly active at the cytocidal level (10-20 nM). In fact, NVP is the most potent antimalarial compound studied in this laboratory to date. These values are within the range of values observed for ATM. While LF has an IC\(_{50}\) of 30-40 nM, the other PI3K inhibitors (GSK212 and PIK 93) had lower activity at the cytostatic level (~100 nM). While Torin2 and PIK 93 had low \(\mu\)M cytocidal activities, GSK212 was determined to have little activity at the LD\(_{50}\) level, with the values ranging from 16 \(\mu\)M in Dd2 to 100 \(\mu\)M in HB3.

Table 5.1. Antiplasmodial Activities of PI3K Inhibitors, Artemether, and Lumefantrine

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (nM) HB3</th>
<th>SEM</th>
<th>Dd2</th>
<th>SEM</th>
<th>LD(_{50}) (nM) HB3</th>
<th>SEM</th>
<th>Dd2</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemether</td>
<td>7.2</td>
<td>0.9</td>
<td>17.1</td>
<td>1.8</td>
<td>16.3</td>
<td>0.4</td>
<td>25.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>33.3</td>
<td>6.9</td>
<td>47.4</td>
<td>18.9</td>
<td>323</td>
<td>52</td>
<td>242.3</td>
<td>13.3</td>
</tr>
<tr>
<td>GSK212</td>
<td>89</td>
<td>48.2</td>
<td>117</td>
<td>17.1</td>
<td>101700</td>
<td>20006</td>
<td>16100</td>
<td>3037</td>
</tr>
<tr>
<td>NVP BGT 226</td>
<td>0.63</td>
<td>0.2</td>
<td>1.05</td>
<td>0.048</td>
<td>17.5</td>
<td>7.5</td>
<td>13.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Torin2</td>
<td>1.00</td>
<td>0.7</td>
<td>2.2</td>
<td>0.25</td>
<td>1875</td>
<td>384</td>
<td>2100</td>
<td>493</td>
</tr>
<tr>
<td>PIK93</td>
<td>129</td>
<td>30.5</td>
<td>217</td>
<td>43.7</td>
<td>2250</td>
<td>250</td>
<td>2033</td>
<td>851</td>
</tr>
</tbody>
</table>
These calculated monotherapy activities were used to probe for possible synergy in drug combinations at both the cytostatic and cytocidal levels using the Chou-Talalay method (C-T) of fixed-ratio analysis, at a ratio of 1:1 based on the activities of the two compounds. For example, the cytostatic combination assay of ATM and LF uses a range of concentrations dictated around their activities, 7 and 33 nM, respectively for HB3. A full growth curve was generated by using both multiples and fractions of those activity values. The ratio between the activity in combination and the activity as a monotherapy generates the fractional inhibitory concentration (FIC). When summed, the FICs for a chosen combination generate the FIC\_index, which is used to assign synergy, additivitiy, or antagonism. Figure 5.4 shows an example of generating the FIC\_index for a combination of GSK212 and LF. The same process is used to analyze these combinations at the cytocidal level, generating FLDs and FLD\_indices, as shown in Figure 5.5 for GSK212 and LF.
**Figure 5.4.** Representative Data of an IC<sub>50</sub> C-T Analysis using GSK and LF.
Figure 5.5. Representative Data of an LD₅₀ C-T Analysis using NVP and ATM.
Most of the studied combinations show additive interactions at the cytostatic level, including the Coartem combination of ATM and LF, as shown in Table 5.2 and Figure 5.6. Synergy is seen in both HB3 and Dd2 parasites for the GSK212/LF combination, as well as GSK212/ATM (HB3 alone), Torin2/LF and PIK 93/LF (both in Dd2 alone). No antagonistic interactions were observed.

**Table 5.2. Average FIC
indices**

<table>
<thead>
<tr>
<th>Combination</th>
<th>FIC&lt;sub&gt;index&lt;/sub&gt;</th>
<th>Average</th>
<th>Assignment</th>
<th>FIC&lt;sub&gt;index&lt;/sub&gt;</th>
<th>Average</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HB3</td>
<td>SEM</td>
<td>Dd2</td>
<td>SEM</td>
<td>HB3</td>
<td>Dd2</td>
</tr>
<tr>
<td>Artemether/Lumefantrine</td>
<td>1.1</td>
<td>0.2</td>
<td>1.1</td>
<td>0.2</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>GSK212/Lumefantrine</td>
<td>1.0</td>
<td>0.2</td>
<td>0.67</td>
<td>0.2</td>
<td>Syn</td>
<td>Syn</td>
</tr>
<tr>
<td>GSK212/Artemether</td>
<td>1.0</td>
<td>0.3</td>
<td>1.1</td>
<td>0.3</td>
<td>Syn</td>
<td>Add</td>
</tr>
<tr>
<td>NVP/Lumefantrine</td>
<td>1.6</td>
<td>0.1</td>
<td>1.5</td>
<td>0.3</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>NVP/Artemether</td>
<td>1.6</td>
<td>0.2</td>
<td>2.0</td>
<td>0.4</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>Torin2/Lumefantrine</td>
<td>1.4</td>
<td>0.4</td>
<td>0.9</td>
<td>0.1</td>
<td>Add</td>
<td>Syn</td>
</tr>
<tr>
<td>Torin2/Artemether</td>
<td>1.3</td>
<td>0.5</td>
<td>1.3</td>
<td>0.2</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>PIK93/Lumefantrine</td>
<td>1.2</td>
<td>0.3</td>
<td>0.81</td>
<td>0.2</td>
<td>Add</td>
<td>Syn</td>
</tr>
<tr>
<td>PIK93/Artemether</td>
<td>1.8</td>
<td>0.6</td>
<td>1.1</td>
<td>0.2</td>
<td>Add</td>
<td>Add</td>
</tr>
</tbody>
</table>
Figure 5.6. Combination FIC \_indices. HB3 (dark grey bars) and Dd2 (light grey bars) parasites were examined for their interactions. Plots are the average of three independent trials run in triplicate (nine determinations total). Error bars are +/- standard error of the mean.

Under cytocidal conditions, many more of the combinations were determined to be synergistic, as shown in Table 5.3 and Figure 5.7. Additive interactions were observed only for ATM/LF and NVP/ATM for HB3 and NVP/LF and PIK 93/LF for Dd2. Unlike the cytostatic analyses, two combinations were observed to be antagonistic under cytocidal conditions, NVP/LF in HB3 and PIK 93/ATM in Dd2. Among the synergistic combinations, the combinations of GSK212 and NVP with both ATM and LF show moderate to high levels of synergy with FLD \_indices ranging from 0.18-0.62. GSK212 is the most potent synergy partner, yielding the lowest FLD \_indices.
Table 5.3. Average FLD<sub>indices</sub>

<table>
<thead>
<tr>
<th>Combination</th>
<th>FLD&lt;sub&gt;indices&lt;/sub&gt; Average</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HB3 SEM</td>
<td>Dd2 SEM</td>
</tr>
<tr>
<td>Artemether/Lumefantrine</td>
<td>1.3 0.4</td>
<td>1.0 0.3</td>
</tr>
<tr>
<td>GSK212/Lumefantrine</td>
<td>0.18 0.04</td>
<td>0.35 0.1</td>
</tr>
<tr>
<td>GSK212/Artemether</td>
<td>0.62 0.1</td>
<td>0.39 0.3</td>
</tr>
<tr>
<td>NVP/Lumefantrine</td>
<td>2.8 0.7</td>
<td>2.0 1.0</td>
</tr>
<tr>
<td>NVP/Artemether</td>
<td>1.4 0.2</td>
<td>0.93 0.3</td>
</tr>
<tr>
<td>Torin2/Lumefantrine</td>
<td>0.28 0.2</td>
<td>0.5 0.4</td>
</tr>
<tr>
<td>Torin2/Artemether</td>
<td>0.46 0.2</td>
<td>0.94 0.4</td>
</tr>
<tr>
<td>PIK93/Lumefantrine</td>
<td>0.90 0.3</td>
<td>1.5 0.03</td>
</tr>
<tr>
<td>PIK93/Artemether</td>
<td>0.85 0.3</td>
<td>2.1 0.4</td>
</tr>
</tbody>
</table>

**Figure 5.7.** Combination FLD<sub>indices</sub>. HB3 (dark grey bars) and Dd2 (light grey bars) parasites were examined for their interactions. Plots are the average of at least two independent trials run in triplicate. Error bars are +/- standard error of the mean.
Previous study of the cellular effects of cytocidal drug treatment of CQ suggests the involvement of the autophagy pathway in parasite death (Gaviria et al. 2013). In eukaryotes, ATG8 positive vesicle formation indicates initiation of autophagy and is downstream from Vps34, a specialized PI3K. As reported previously, an ortholog of Vps34 has been identified in *P. falciparum*, the only PI3K identified to date. Radial redistribution of puncta in also occurs in response to ATM at LD$_{50}$ dose (Fig. 5.8B) compared to IC$_{50}$ dose (Fig. 5.8A). The application of Coartem (ATM+LF) induced an even greater response (Fig. 5.8C). Interestingly, both NVP-BGT226 and GSK-2126458 blocked increased autophagosomal trafficking induced by ARTs, CQ and LMF (Fig. 5.8D,E). Similar to other eukaryotes, this suggests a role for PfVps34, as the sole PI3K, and PI3P in the parasite autophagy response and also suggests that targeting this response is a common theme for synergistic drug combinations identified in this study.
Figure 5.8. Analysis of Autophagosomal Body Puncta Formation and Trafficking in Response to Environmental and/or Pharmacological Stress. Imaging and quantification of PfAtg8 containing puncta within parasite infected erythrocytes under differential interference contrast (panel 1), anti-PfAtg8 peptide antibody imaging (ex: 450-490, em: 500 to 550)(panel 2), DAPI nuclei staining (ex: 340-380, em: 450 to 490)(panel 3), a merged image of all three views (panel 4) following a 6 hour exposure to (A) ATM at the defined IC_{50} value. (B) ATM at the defined LD_{50}
value. (C) ATM and LF at their respectively defined LD\textsubscript{50} values. (D) ATM at the defined LD\textsubscript{50} value and GSK-2126458 at 102 μM. (E) ATM at the defined LD\textsubscript{50} value and NVP-BGT226 at 24 nM. Scale bar equals 5 μm.

5.3. Discussion

Much of the early laboratory results of ATM/LF combination, commonly known as Coartem, are either not available outside China, or are only published in Chinese, making examination of the path to WHO support difficult. Studies from 1979 report antimalarial activity, and “preclinical studies” claim to show synergy between LF and ATM. Unfortunately, this claim is not supported in the WHO report with a verifiable citation (Olliaro \textit{et al.} 1995). Other sources that reference synergy typically include discussion of the pharmacokinetic properties of ATM and LF and results for parasite clearance in animal models (White \textit{et al.} 1999; Makanga \textit{et al.} 2009). Unfortunately, the lack of a firm definition for “drug synergy” in malarial science has led to its use in conditions like complementary pharmacokinetics, which are not proper synergy as defined mathematically. I have therefore revisited this critical issue using C-T methods.

The combined activity of partner drugs being greater than a monotherapy alone does not mean the combination is necessarily synergistic; such a result could be caused by additive or even slightly antagonistic interactions. A clear mathematical definition of synergy vs. additivity vs. antagonism is needed to avoid confusion. By definition, synergy is activity that is greater than that of an additive interaction; antagonism is activity that is less than additive. The typical way to describe the all-important additive effect is by envisioning the mixing two unknown drug solutions that are actually comprised of the same compound. The resulting data and combination
analysis is, by definition, additive. Additionally, synergy requires the presence of two active compounds. If the presence of an inactive compound augments the activity of an active drug, this is not synergy; it is potentiation (Chou 2006; Chou 2010). It is important to remember that in situations like the one described, just because a pair of compounds are not mathematically synergistic does not mean that they are not a viable treatment option. An additive drug combination may be better clinically than a synergistic one, depending on additional factors such as pharmacokinetics, pharmacodynamics, and therapeutic windows for the compounds.

Reports of ART tolerance in the Thai-Cambodian border region (Dondorp et al. 2009) and the generation of temporary tolerance to LF in vitro (Mwai et al. 2012) suggest that novel drug combinations must be explored before Coartem begins to lose efficacy. In addition to the isobolograms focused on the ATM and LF combination, as described earlier, other antimalarial combinations have been studied. These include: atovaquone/proguanil, atovaquone/DHA (Fivelman et al. 2004); 9-epi-ART/ART, artemisitene/ART, 9-epi-ART/ATSU, artemisitene/ATSU (Suberu et al. 2013). The Chou-Talalay method, which was used in the Thriemer and Wong studies detailed previously, has been also been used to investigate the interaction of ART with other natural products isolated from ART tea (Suberu et al. 2013), the analysis of possible drug partners emetine/DHA (Matthews et al. 2013), tapsigargin/ART, tapsigargin/OZ227 (Abiodun et al. 2013), and the interaction of chalcone derivatives and ART (Battacharya et al. 2009).

High-throughput screening of a library of compounds identified a series of PI3K inhibitors for further screening. A selected set of these compounds, GSK212, NVP, Torin2 and PIK 93, were partnered with both LF, ATM, and quinolines (preliminary data not shown) in
combination analyses at cytostatic and cytocidal levels. Overall, most pairings under cytostatic conditions were additive, including the Coartem pairing of ATM and LF. Under cytocidal conditions, most were synergistic, with NVP/LF being the worst overall pairing under those conditions. ATM/LF was additive under these conditions as well. Interestingly, the ATM/LF combination, when studied for its effects on ATG8 puncta distribution shows an increase in ATG8-positive puncta, suggesting that the presence of two sources of oxidative stress have increased the cellular response via autophagy.

The most potently synergistic PI3K with ATM and LF was GSK212, even with its moderate cytostatic activity and low cytocidal activity. GSK212 is a potent inhibitor of both human class I PI3Ks and the mammalian target of rapamycin (mTOR) neither of which have direct orthologs that have been identified in *P. falciparum*. It has been shown to have low picomolar inhibitory activity against PI3Kα isolated from cancer cell lines. The selectivity for class I and IV PI3Ks over other protein kinases is in excess of 200 fold (Knight *et al.* 2010). In mammalian cell lines, GSK212 has shown both growth inhibitory and cell kill effects through inhibition of the PI3K/mTOR signaling pathway (Leung *et al.* 2011). Although class I and IV PI3Ks have not been identified, an alteration of the ATG8-puncta phenotype resulting from ATM treatment suggests that GSK212 is active against the Vps 34 PI3K within the *Plasmodium* autophagy pathway.

NVP is another dual PI3K/mTOR inhibitor previously studied in cancer cell lines. NVP has been observed to inhibit p110α, β, δ, and γ isoforms at nanomolar concentrations, as well as mTOR (Markman *et al.* 2012). It has been shown to have growth inhibitory and cytocidal effects. The absence of DNA fragmentation in these studies suggests that it does not promote apoptosis;
however the presence of autophagic vesicles following treatment has been observed, which was hypothesized to be the cell-death pathway following treatment (Chang et al. 2011). Clinical investigation of its use against cancer was halted, as the plasma concentration never reached therapeutic concentrations within the tolerated dose window (Markman, et al. 2012). As a monotherapy, NVP shows antiplasmodial activities in the same range of ART-based compounds (low nM). In combination with ATM and LF at cytostatic doses, the result was an additive designation, with NVP/ATM low additive to synergistic and NVP/LF high additive to antagonistic under cytocidal conditions. As in the case for GSK212, the presence of ATG8-positive puncta resulting from ATM treatment is reduced when treated with NVP and ATM at cytocidal doses. Although NVP is selective for class I PI3Ks and mTOR, there must be a target for it in the parasite’s autophagy cascade, likely the Vps34 PI3K.

Torin2 inhibits of various phosphatidylinositol kinases at all concentrations, including PI3Kα. However, it is over 800-fold more selective for mTOR than PI3Ks. Treatment of cancer cell lines with Torin2 over the course of 72 hrs showed a dose-dependent increase in autophagosomes, while higher concentrations resulted in signs of cellular apoptosis (Liu et al. 2013). Torin2 has been studied in both liver and erythrocyte stages of Plasmodia, with hundreds nanomolar activity against liver stages and low single-digit nanomolar activity against the erythrocyte stages (Hanson et al. 2013). Its effectiveness against both stages shows that host-cell mTOR is not involved, as red blood cells do not contain a functioning mTOR. Torin2 treatment results in altered export of proteins to the parasitophorous vacuolar membrane in liver cells, but the effect of Torin2 treatment on intraerythrocytic parasites is not known (Hanson et al. 2013). Combining Torin2 with ATM and LF results in low additivity to slight synergy under cytostatic
conditions, while all combinations are solidly synergistic under cytocidal conditions. Further investigation is still needed to probe Torin2’s impact on the autophagy response to treatment with known antimalarials. The antiplasmodial activity, as well as the previously-observed alteration of PVM trafficking suggests that Torin2 is able to inhibit the Plasmodium autophagy cascade.

PIK 93 is a PI3K inhibitor that has been noted as potent against p110γ isoforms, as well as inhibiting other isoforms. However, it is a highly potent inhibitor of human PI4 kinases, particularly the IIIβ isoform which it is 100-fold more potent against as compared to human PI4KIIIα (Knight et al. 2006; Balla et al. 2008). In fact it is used as a method of delineating the activity of one human enzyme versus the other (Tóth et al. 2006). PI4Ks are involved in cellular signaling and trafficking (particularly calcium signaling) (McNamara et al. 2013; Knight et al. 2006). PI4K inhibitors have recently been studied for their antimalarial activity (McNamara et al. 2013). The identified target, PfPI(4)K is deemed a functional complement to yeast Pik1, a PI(4)KIIIβ that regulates membrane trafficking involved in the membrane alteration required for the completion of the asexual growth cycle. This target was identified through the use of other inhibitors, imidazolepyrazine and quinoxaline, but not PIK 93 (McNamara et al. 2013). Partnering PIK 93 with LF and ATM results in interesting differences between CQS and CQR parasites. Under cytostatic conditions, HB3 parasites showed lower additivity as compared to Dd2, which had synergy when PIK 93 was paired with LF. Under cytocidal conditions, HB3 showed synergy for both combinations, while the Dd2 was additive with LF and antagonistic with ATM. Examining the ATG8 puncta phenotype of these combinations can shed light on
whether or not PIK 93 combinations result in an altered autophagy cascade, or if its activity is due to inhibiting PI4Ks.

Autophagy is both a recovery response to toxic shock and a programmed cell death pathway. When compounds such as ATM and LF cause oxidative stress and damage, the autophagy pathway can help the parasite withstand the shock and remain viable, or if it progresses too far, it can die. In combination therapy, it is possible for one drug, like GSK212 or NVP, to interfere with the life-saving aspect of autophagy, preventing the restoration of cellular viability. It is also possible for the two partner drugs to both promote autophagy, and cause it to run out of control and kill the parasite, which may be the case for ATM and LF, which show increased ATG8 puncta. Further exploration of the interactions between PI3K inhibitors and known antimalarials could result in a new very attractive therapy, particularly if the human PI3K inhibitors identified here are optimized against PfVps34 or other kinases in the *Plasmodium* genome.

5.4 Acknowledgements

High-throughput robotics screening of the library of compounds was performed by the Thomas Laboratory at the National Center for Advancing Translational Science (NCATs), National Institutes of Health (NIH), Rockville, MD.

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antiplasmodial and combination assays, as well as the cytosolic calcium measurements and immunofluorescence assays.
Recent focus on cytocidal drug effects vs. *Plasmodium falciparum* malarial parasites has suggested that multiple drug targets may exist within the parasite. The prevailing theory of CQ’s mechanism of action, that it interferes with the Hz crystallization pathway, may not fully explain drug action at higher doses (Gorka *et al.* 2013a; Paguio *et al.* 2011; Cabrera *et al.* 2009a; Gligorijevic *et al.* 2008). By modeling the ability of quinolines like CQ and QN to interfere with the crystallization of beta-hematin, the measured activity indeed correlates with cytostatic (IC$_{50}$) activity, but not with cytocidal (LD$_{50}$) activity (Gorka *et al.* 2013a,d). Similarly, compounds that do not inhibit beta-hematin, such as epi-QN and epi-QD, and also do not alter the pH-dependent solubility of FPIX, have low IC$_{50}$ activity, but have LD$_{50}$ activities closer to those of their 9-epimers (Gorka *et al.* 2013d). Additional observations of altered daughter nuclei distribution, reduced viability of the next cell cycle (Gligorijevic *et al.* 2008), and the ability of resistant parasites to survive concentrations of CQ in the DV that are toxic to susceptible parasites suggest that the targets of cytocidal drug treatment, particularly CQ, are different from that of cytostatic doses (Cabrera *et al.* 2009a).

QTL analysis of the cytocidal resistance phenotype does identify the region of chromosome 7 that contains the *pfcrt* loci, but also identifies regions that include genes that code for proteins involved in cellular pathways that include lipid metabolism, proteosome function, and vesicle transport, all of which could be implicated in the autophagy programmed cell death pathway (Gaviria *et al.* 2013). Visualizing the distribution of ATG8-positive puncta following
cytocidal CQ treatment has identified an altered autophagic response in CQR parasites compared to CQS (Gaviria et al. 2013). The components of this pathway are outside of the DV, supporting the hypothesis that DV targets are applicable under cytostatic conditions, but additional cellular targets are impacted under cytocidal conditions.

In order to identify possible targets of cytocidal CQ, a novel ABPP probe was synthesized based on the framework of a previously used probe, AzBCQ (Lekostaj et al. 2008). By incorporating bioorthogonal click chemistry, the experimental versatility of the new probe exceeds that of AzBCQ. Labeling the probe with an azide-coupled fluorophore in fixed cells, followed by examination through fluorescence microscopy, shows that at IC$_{50}$ concentrations, the staining is contained within a smaller area of the parasite, typically within close proximity to the optically-dense Hz, possibly through labeling of PfCRT. Although no staining is observed within the DV, this may be due to the weak interactions of CQ and FPIX compared to protein binding. The association constant of CQ and FPIX were determined to be only about 4x10$^5$ M$^{-1}$ (Dorn et al. 1998). Additionally, if the photoaffinity tag is not in close proximity to a heme C-H sigma bond, it may not undergo insertion before the nitrene decays. CQ has been observed to bind to FPIX in both its monomer (de Dios et al. 2003) and µ-oxo dimer (Leed et al. 2002) forms. CQ binds monomer FPIX through a dative bond between the quinoline nitrogen and the iron center (de Dios et al. 2003), leaving the aliphatic side chains oriented away from the porphyrin ring. When CQ binds to µ-oxo dimer FPIX, the aromatic quinoline ring is oriented over the porphyrin ring, with the aliphatic side chains oriented in a plane approximately parallel to the other half of the porphyrin ring (Leed et al. 2002). The distance between the side chain and the porphyrin, however, ranges between 4.2 and 5.8 Å, which could be too large of a distance for
the nitrene to efficiently insert (Leed et al. 2002). Since FPIX is a planar molecule, the aliphatic side chain that contains the photoaffinity tag is probably not close enough to successfully crosslink. Alternatively, however, when the probe is bound within a binding pocket of a protein, the reactive nitrene could be in closer proximity, making it more likely to insert into a bond within the pocket.

Under cytocidal dosages, AAzCQ staining is observed more intensely and more widely distributed throughout the parasite cytosol. Efficient competition with CQ supports that this staining is CQ-specific, and not an artifact of non-specific labeling. If the PfCRT-mediated transport of CQ out of the DV away from the FPIX target is more applicable at cytostatic concentrations, it may mean that at cytocidal doses, the targets are spread throughout the cell, and not localized to the DV and its membrane. Autophagy, as a cellular survival and death pathway, would occur throughout the cytosol, clearly evident in the ATG8 staining of parasites following starvation or cytocidal CQ treatment in Gaviria et al. (2013).

If cytocidal targets are outside the DV, then alterations or mutations in these targets would be relevant for elevated LD$_{50}$ values in CQR parasites (Gaviria et al. 2013). Initial examination of the binding of AzBCQ to protein targets in HB3 and Dd2 parasites shows some promising possible protein hits. In the CQS HB3, labeling of proteins such as heat shock protein 70 and a subunit of the proteosome, suggest that protein degradation and proper folding could be a response to CQ treatment, possibly in responding to the toxic insult of CQ treatment. Neither of these proteins were identified in the CQR Dd2 parasites, which identified a calcium-binding protein from the endoplasmic reticulum and the two isoforms of actin present in $P. falciparum$. This could mean that alterations in the processes of cellular response to toxic insult could be
contributing to the elevated LD$_{50}$ in Dd2 parasites. This is consistent with the altered autophagy response noted in Gaviria et al. (2013). An alternative to this hypothesis is that the toxic insult of cytocidal CQ that results in the initiation of processes involving heat shock protein 70 and the proteosome in HB3 parasites does not also initiate these responses in Dd2 parasites due to a possible alteration of the pathway. Further investigation of cellular targets and the nature of CQ binding could further elucidate the involvement of heat shock protein 70 and the proteosome.

Labeling parasites for proteomic analysis with both AAzCQ and AzBCQ at the respective LD$_{50}$ values of HB3 and Dd2 shows differential labeling between CQS and CQR strains that is successfully competed by CQ. This labeling is not necessarily the same between the two probes. The large size of the biotin moiety of AzBCQ might either prevent successful binding to CQ targets or increase the non-specific interactions with off-target proteins. If the mechanism of cytocidal resistance relies on reduced ability of CQ to bind to its cytoplasmic target, one would expect that labeling of the protein would be reduced or eliminated in resistant strains as compared to susceptible ones. If, however, resistance arises due to enhanced transport away from the potential target, much in the way mutant PfCRT removes CQ from FPIX targets at cytostatic concentrations, one would expect that the transport protein would then be labeled more so in the resistant strains than the susceptible. Differential labeling observed by labeling parasites at their respective cytocidal doses leads to both the presence of enhanced labeling in susceptible strains as well as the presence of labeled proteins in resistant strains that are not labeled in susceptible ones. Further study of the identity of these proteins, currently underway, should help to shed light on the nature of the differential labeling targets. In order to confirm that the protein does indeed bind CQ, it can be expressed in a yeast system for further study of a purified protein.
though use of the photoaffinity probes and possibly, if transport of CQ is of concern, the use of fluorescently-tagged CQ analogs (Cabrera et al. 2009b).

Understanding resistance needs to be partnered with development of new therapies that are effective against resistant strains. In order to prevent the rise of resistance, combination therapies are now used (Greyer, ed. 2001; Hastings et al. 2002). Instead of mutating to avoid the toxic effect of one compound, the parasite must now evolve to avoid the toxic effect of both, as well as any synergistic effects that may come from their interactions. High-throughput screening performed at NCATS has identified multiple classes of compounds that show synergy with currently-used antimalarial drugs. One of these classes is the PI3K inhibitors. Analysis of PI3K inhibitors in combination with ATM and LF has shown mostly additive interactions when studied under cytostatic conditions, but shows high levels of synergy under cytocidal conditions. Preliminary analyses of combinations of these PI3K inhibitors with quinolines show high levels of synergy, particularly in combination with CQ, which will be studied further.

If autophagy is involved in response to cytocidal drug treatment, it is not, then, coincidental that PI3K inhibitors would augment the activity of antimalarial compounds since PI3K activity is essential to induction of autophagy (Brennand et al. 2011). As autophagy is a cellular survival pathway, inhibition of the proper function of this pathway would prevent the cell from recovering. When these combinations are examined for their effects on ATG8-puncta distribution, the ATM/LF pairing shows a possible increase in puncta, but upon pairing ATM with either GSK212 or NVP BGT 226, the puncta response is severely diminished. Autophagy is both a cell-survival and cell-death pathway. After the toxic stress of ATM, the parasite could initiate autophagy as a way to survive. By inhibiting Vps 34 (the only identified PI3K in
Plasmodium), PI3K inhibitors would prevent the parasite from utilizing autophagy to survive, and therefore die.

In the same manner, it is possible that CQ acts as its own partner drug by having multiple effects that are likely dose-dependent. After suffering from the oxidative stress generating from the interaction of CQ with FPIX within the DV, the parasite appears to initiate autophagy in order to survive (Gaviria et al. 2013). But, as is observed in cancer cell lines, CQ has the characteristic of being lysosomotropic, meaning that it penetrates the lysosome and accumulates there by acid-trapping, as CQ is a weak base. The net effect of this lysosomotropic activity is collapse of lysosome pH gradients, thereby preventing the binding of autophagosomes with lysosomes to complete autophagy (Seitz et al. 2012). Perhaps related to this idea, in Hoppe et al. 2004, study of the effect of CQ and ART on the trafficking of endocytotic vesicles was examined. Vesicles containing host Hb were observed to increase in number following treatment with either drug, possibly due to the inability of the vesicles to fuse with the DV. Similarly, pairings like ATM/LF could interact by accelerating toxic stress or inhibiting autophagy downstream, possibly by preventing the fusion of autophagosomes with the DV. Further exploration of the pairing of PI3K inhibitors with currently-used antimalarials would benefit from expressing the binding and catalytic sites of Vps 34 in a yeast model system and examination of the binding of inhibitors and their effect on the enzyme’s activity. Optimization of compounds targeting PfVsp 34 could lead to even greater synergistic interactions, and possible new combination therapies.
Understanding resistance, its rise and the cellular mechanisms that underlie it, as well as developing novel combination therapies active against resistant strains are key tools to use in the battle against the global health crisis that is malaria.
7.1 Quantification of Perchloroethylene Residues in Dry Cleaned Fabrics

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Quantification of perchloroethylene (PCE) residues in dry cleaned fabrics. *Environmental Chemistry and Toxicology*. 2011, 30(11), 2481-2487. Copyright © 2011 SETAC. Used with Permission.

7.1.1. Introduction

Concerns over ozone depletion led the US Environmental Protection Agency (EPA) to recommend a switch in dry-cleaning solvent from 1,1,1-trichloroethane to the more photostable, yet more toxic perchloroethylene (PCE) and trichloroethylene (Roy *et al.* 2002). As of 1994, more than 90% of clothing dry cleaners in the US and Europe used PCE, raising concerns over its toxic effects to dry cleaning workers and to the surrounding environment (Aggazzotti *et al.* 1994). Studies have detected hazardous levels of PCE in ground water and drinking water, as well as in the air of buildings containing dry cleaners, and in areas where dry cleaned clothes are stored (Aggazzotti *et al.* 1994; Furuki *et al.* 2000).

PCE has a longer *in vivo* lifetime than other organic solvents, and can be absorbed through inhalation, mouth, or skin contact, preferring to distribute to hydrophobic tissue, particularly adipose tissue (Aggazzotti *et al.* 1994). Toxic effects that have been observed in animal models include fatty liver degeneration, liver enlargement and abnormal function,
necrosis, decreased levels of cellular adenosine triphosphate (ATP), lesions on the liver and kidneys, and cancer (Yoshioka et al. 2002; White et al. 2002). Although controversial, human PCE exposure has been suggested to be linked to elevated risk of cancer, infertility, spontaneous abortion, and depression of the central nervous system (Aggazzotti et al. 1994; Yoshioka et al. 2002; Coyle et al. 2005). Conflicting carcinogenic effects in animal models, as well as some inconclusive epidemiological results have resulted in the EPA labeling PCE as a possible (class C) or probable (class B2) carcinogen and the International Agency for Research on Cancer (IARC) listing PCE as a probable (class 2A) human carcinogen (Brand et al. 1999; Poli et al. 2005).

The vast majority of PCE exposure studies have focused on inhalation of vapors in occupational environments. In only two studies to our knowledge have PCE residues in dry cleaned fabrics been examined. Brand and colleagues examined levels of residual PCE in dry cleaned acetate fabric, focusing in particular on whether pressing dry cleaned acetate increased worker inhalation risk (Brand et al. 1999). In this work, Brand and colleagues used gas chromatography with a flame ionization detector to quantify PCE based on retention time (Brand et al. 1999). Statistically-significant differences were seen between several dry cleaners, with all values within a four-fold window, and pressing was determined to lower PCE retained on the fabric (Brand et al. 1999). Kawauchi et al examined PCE residues in a few fabric types dry cleaned at several different establishments, using a hexane extraction protocol with gas chromatographic detection (Kawauchi et al. 1989). They also reported a limited number of air concentrations in storage outlets and consumer’s homes (Kawauchi et al. 1989).
In this study we develop an improved gas chromatography/mass spectrometry approach to unambiguously quantify the level of residual PCE extracted with a non organic solvent (methanol) from four common fabrics (wool, cotton, polyester, and silk). For the first time, PCE is analyzed vs. number of drug cleaning cycles from seven different commercial locations. The advantage of using mass spectroscopy over absorbance, flame ionization or electron capture is that the mass spectrum simultaneously and unambiguously verifies the chemical identity of the compound(s) being eluted at various GC retention times.

The results are provocative and suggest further work aimed at PCE surveillance for different fabric types is likely warranted.

7.1.2. Materials and Methods

Materials

Wool, polyester, cotton, and silk were purchased as 1.47 by 0.91 m bolts from Jo-Anne Fabrics in either Manassas, VA or Leesburg, VA. Fabric specifications were: Papyrus 100% white wool suiting manufactured in China, 100% white polyester or white satin taffeta manufactured in Japan, 100% white cotton manufactured in the United States, and 100% white Dupioni silk manufactured in India. Perchloroethylene was purchased from Flinn Scientific (Batavia, IL). High-resolution GC-grade methanol, 5 mL class A volumetric pipettes (± 0.01 mL tolerance), Whatman 70 mm diameter qualitative filter papers, Target LoVial, 2 mL clear glass I-D GC vials, and borosilicate glass scintillation vials were purchased from Fisher Scientific (Newark, DE).
Fabric dry cleaning

Each fabric bolt was cut into approximately 5 cm$^2$ swatches and attached beneath the lining of a men’s suit jacket. The lining at the bottom of the jacket was gently undone and the four cloth swatches sewn along one edge onto the interior of the pocket lining, ensuring it was both secure and undetectable. The lining opening was re-sewn and the jacket subjected to a single dry cleaning cycle, without pressing. For multiple cycle tests, the jacket was kept intact and returned to the same dry cleaner to undergo additional cycles of cleaning. Upon removal from the jacket lining, the cloth swatches were immediately transferred to a sealed, zip-top plastic bag and stored at -20 °C until analysis.

Extraction of PCE from fabric samples

A 0.5 x 2.0 inch swatch of 100% wool, polyester, cotton, or silk dry cleaned for zero (negative control) or 1 cycle at each of 7 Northern Virginia dry cleaners was placed in a capped 20 mL borosilicate glass scintillation vial. Additionally, the same size swatch of each type of fabric subjected to multiple dry cleaning cycles (2 to 6) at dry cleaning establishment A was similarly prepared. Five mL of GC-grade methanol was added to each vial using a volumetric pipette and the vial re-capped. Extraction was allowed to proceed at room temperature for 24 h (time dependence of extraction is shown in “supplemental data”, Fig. 7.1 at which time the extract was passed through standard qualitative filter paper and transferred to a clean scintillation vial. Extracts were stored at 4 °C while awaiting GCMS analysis.
Figure 7.1. PCE extraction from wool fabric dry cleaned at establishment F vs. time. Fabric samples were extracted for 5 min, 15 min, or 1, 12, 24, and 48 h as described in the Materials and Methods section. Three replicates were run for each time point except 12 h (n = 2). The data were averaged; error bars represent standard error of the mean.

GCMS analysis

Samples were run on a Varian 3900 gas chromatograph with a model 2100 ion trap mass spectrometer using electron-impact ionization. The column was an Agilent “HP-5” column with a (5% phenyl)-methylpolysiloxane stationary phase, with a diameter of 0.25 mm and a length of
A temperature profile was generated to ensure resolution of PCE. The injection port was maintained at 250 °C. Following a solvent delay of 2.75 minutes, the oven temperature of 50 °C was held for 5 minutes, increased to 120 °C at 10 °C per minute, and held at that temperature for 2 minutes, for a total run time of 17 minutes. This temperature profile resulted in a PCE retention time of 5 minutes. In order to minimize instrumental error, a set of PCE standards were run before each set of samples.

Calculation of PCE levels in fabric extracts

Standardized solutions of PCE: 10, 50, 100, 500 μM and 1 mM, diluted in GC-grade methanol were used to calculate PCE concentration in the fabric methanol extracts. A negative control, comprised of the methanol with no PCE, was also run. Each day fabric samples were analyze, three injections of each sample were analyzed, integrating the PCE peak at a retention time of 5 minutes, for each injection, using Varian MS Workstation software. Peak area for three injections of each PCE standard were averaged, and a standard curve generated by plotting averaged area versus the known concentration.

Once the concentration of PCE in a fabric methanol extract was calculated using a standard curve obtained under the same conditions, the injection volume (1 μL) and the known area of the fabric sample were used to calculate the amount of PCE in the fabric in units of nmol/cm². Fabric samples were analyzed in triplicate; the mean and the standard error of the mean were calculated.
7.1.3. Results

In order to develop a simple method for quantifying PCE residue in common clothing fabrics, we inspected several possible solvents for extraction. One in particular that appeared useful to us was pure methanol, since PCE is miscible with methanol, does not degrade common fabrics, is an appropriate solvent for GCMS analysis, and is purged from the column in approximately 3 minutes, which is a suitable solvent delay.

Figure 7.2A shows representative chromatograms of methanol, and two known concentrations of PCE in methanol. The truncated peak at about 2.5 minutes is the tail end of the (very large) methanol solvent peak. Degradation of the column stationary phase led to several small peaks including a residual peak near 5 minutes in the methanol blanks, which was included in calculation of the standard curve. Integration of the prominent PCE peak at 5 minutes increased with increasing concentration. Figure 7.2B shows a representative standard curve calculated using integrated peak area. A strong linear correlation was observed, thus linear regression was used to quantify fabric sample extracts.
Figure 7.2. Standard curve generation using known PCE peak area vs. concentration. Before each set of samples, standard concentrations of PCE in MeOH were run, and their chromatographic peaks integrated to produce a standard curve used in concentration calculations (see Materials and Methods) A. Chromatograms of a methanol blank (dotted line), as well as 50 μM (solid line) and 500 μM (dashed line) PCE standards. The peak at about 5 minutes is integrated. B. Integrated peak areas (in millions of ions impacting the detector, or millions of counts) were plotted versus known concentration of the standards. The data points shown are the
average of three replicates, with the error bars representing the standard error of the mean (note: error bars fall within the symbols).

Figure 7.3. Representative chromatograms of control (non-dry cleaned) fabric. Swatches of each fabric type subjected to no dry cleaning were extracted and analyzed as described in the Materials and Methods section. Results show only the stationary phase peak seen in the methanol blanks (Figure 7.4A) and are devoid of the PCE peak at 5 min.
Figure 7.4. Representative mass spectra of a 1 mM PCE standard (A) and a polyester extract sample from dry cleaner A (B). The mass spectrum associated with the integrated peak verifies the presence of PCE. Peaks labeled with an asterisk represent the loss of 3, 2, and 1 chlorine atoms expected via this method of detection.
Methanol extractions on control (non-dry cleaned) swatches of each fabric showed only the stationary phase peak seen in the methanol blanks (Figure 7.3), which is calculated as no detectable PCE concentration. The lack of a significant peak area detected for control fabric samples supports that the peak area increase for test fabric samples (see below) is indeed due to PCE, not to fabric contaminants (see also discussion of Fig 7.4, below).

Even though identical peak shape and retention time would be strongly suggestive that PCE is indeed extracted from these samples (see below), we confirmed the presence of PCE by examining the mass spectrum of each sample. Figure 7.4. shows representative mass spectra of the 1 mM PCE standard (Fig. 7.4A), and a polyester sample from dry cleaner A (Fig. 7.4B). The molecular ion peak is seen at \( m/z = 166 \), with peaks associated with successive loss of chlorine atoms at \( m/z = 131 \) (trichloroethylene), 94 (dichloroethylene), and 59 (monochloroethylene). The presence of this molecular ion peak fingerprint confirms of the presence of PCE. Degradation of the column stationary phase was observed at very low PCE concentrations, with the largest peak at \( m/z = 207 \).

Figure 7.5 shows representative chromatograms of all four fabrics from two of the seven dry cleaners. Integration of the PCE peaks at a retention time of 5 minutes yielded areas that varied among fabric type as well as between different dry cleaners. The amount of PCE was calculated using a standard curve (e.g. Figure 7.2B), the injection volume, and the measured area of the fabric swatch. Data from three independent extractions for each fabric from each dry cleaner were averaged, and the standard error of the mean calculated, as shown in Table 7.1 and Figure 7.6.
Figure 7.5. Representative chromatograms of fabric samples. The peak with a retention time of 5 minutes (marked with an asterix) harboring PCE was integrated to calculate the concentration of PCE in the methanol extract. Note: dry cleaner “D” identifies itself as a “green” dry cleaner, hence peak integrated intensity is zero, after subtraction of trivial absorbance at similar retention seen for MeOH blank (see also Table 7.1).

Table 7.1. Calculated Residual PCE from Seven Dry Cleaners. The amount (in nmol) of PCE per unit area (cm²) from the four different fabric types is shown as the average of three separate extractions, with the standard error of the mean shown in parentheses. Note: dry cleaners “D” and “G” identify themselves as “green” dry cleaners. Correspondingly, samples from D and G show no PCE. “ND” - Not Detected.

<table>
<thead>
<tr>
<th>Amount of Perchloroethylene per Area (nmol/cm²)</th>
<th>Cotton</th>
<th>Polyester</th>
<th>Silk</th>
<th>Wool</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dry Cleaner A</strong></td>
<td>12.91 (0.62)</td>
<td>56.76 (5.55)</td>
<td>ND</td>
<td>47.58 (7.31)</td>
</tr>
<tr>
<td><strong>Dry Cleaner B</strong></td>
<td>10.70 (0.44)</td>
<td>28.03 (0.61)</td>
<td>ND</td>
<td>16.43 (1.71)</td>
</tr>
<tr>
<td><strong>Dry Cleaner C</strong></td>
<td>14.87 (1.54)</td>
<td>39.27 (4.23)</td>
<td>ND</td>
<td>25.23 (1.58)</td>
</tr>
<tr>
<td><strong>Dry Cleaner D</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Dry Cleaner E</strong></td>
<td>21.74 (2.32)</td>
<td>50.06 (3.55)</td>
<td>ND</td>
<td>31.37 (3.03)</td>
</tr>
<tr>
<td><strong>Dry Cleaner F</strong></td>
<td>24.66 (1.68)</td>
<td>53.28 (7.11)</td>
<td>ND</td>
<td>36.85 (1.76)</td>
</tr>
<tr>
<td><strong>Dry Cleaner G</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 7.6. The amount of residual PCE per unit area of fabric. All four fabrics were tested from each dry cleaner: dry cleaner A (solid bars), B (dashed bars), C (open bars), D (diamond bars), E (striped bars), F (dotted bars), G (checkered bars). No PCE was detected for dry cleaners D and G, hence their bars are not visible. The concentrations are the average of three extractions, with error bars representing standard error of the mean.

Interestingly, for all seven dry cleaners, the silk samples did not show any detectible levels of PCE (Fig. 7.6). Also, dry cleaners “D” and “G” showed no detectible levels of PCE in any of the fabrics (Fig. 7.6, Table 7.1). Not coincidentally, these two dry cleaners advertise themselves as “green” dry cleaners, meaning, presumably, no PCE is used in the cleaning.
process. The chromatogram of dry cleaner D did not show any significant additional peaks relative to MeOH blank, as seen in Figure 7.5 (see also Fig. 7.2A, dotted line). The lack of any other compounds extracted by the methanol suggests that this dry cleaner likely employs supercritical carbon dioxide in the cleaning process. On the other hand, dry cleaner G showed no PCE peak at 5 minutes, but did reveal a peak at about 13 minutes with a mass spectrum consistent with a long-chain alkane (peaks at \( m/z = 57, 71, 85, \) and 169, data not shown). With a molecular ion of \( m/z = 282 \), the compound may be one or many isomers of eicosane, \( \text{C}_{20}\text{H}_{42} \), which is a component of alternative petroleum-based hydrocarbon mixtures (EPA 2002). Such dry cleaning solvents are typically comprised of varied composition linear and branched saturated alkanes and cycloalkanes (EPA 2002).

We next wondered whether PCE residue remaining from traditional dry cleaning establishments is volatilized from fabric samples, and if so, at what rate. Wool samples from dry cleaner A were left exposed to open air at room temperature, as well as wrapped under dry cleaner-provided plastic. No significant differences were seen between the two environments (Figure 7.7). Over the course of 7 days, the amount of PCE found in either wool sample from either environment was reduced by about half (Figure 7.7).
Figure 7.7. Volatilization of residual PCE from wool fabric dry cleaned at establishment A at ambient room temperature either in open-air (solid circles) or under dry cleaner-provided garment plastic (open circles). A 1.3 x 5.0 cm swatch was placed on filter paper and allowed to stand either exposed to open-air or loosely folded in dry cleaner-provided garment plastic for 1, 2, 3, 4, 5, and 7 days. Samples were left undisturbed on the laboratory bench under normal airflow conditions. Each was then extracted with MeOH for 24 h and extract analyzed for PCE content as described in Materials and Methods. Three replicates were analyzed for each time point, the data were averaged; error bars represent standard error of the mean.
Figure 7.8. Accumulation of PCE within different fabric types over multiple dry cleaning cycles. Single samples of fabric were dry cleaned 1 to 6 times: cotton (filled circles), polyester (open circles), wool (triangles). Three replicates were run for each sample except wool at 4 cycles (n = 4) and 5 cycles (n = 2). The data were averaged; error bars represent standard error of the mean.

Finally, we were curious to know whether multiple dry cleaning runs would accumulate PCE. Samples were dry cleaned at the same establishment for up to 6 successive cycles (Figure 7.7). The amount of PCE detected was found to increase, but in a fabric dependent fashion. Cotton was found to plateau after about 2 cycles, and to a lower level than either polyester or wool. Polyester levels plateaued after 3 cycles, to a lower level than wool. Wool levels increased in each cycle, surpassing both polyester (at 3 cycles) and cotton (at 2 cycles), and may not have plateaued, even after 6 dry cleaning cycles.
7.1.4. Discussion

Our analysis of PCE extracted from conventionally dry cleaned fabric shows surprisingly high levels in cotton, polyester, and wool, ranging from 10 to 56 nmol/cm². After a single cycle of dry cleaning, polyester showed the highest retention, followed by wool and cotton. From all seven dry cleaners, no silk samples showed detectable PCE. Two dry cleaners, which advertised as “green”, had no detectible levels of PCE in any fabric, but one did show evidence of long-chain alkanes that are found in “green” petroleum-based hydrocarbon solvents (EPA 2002) in a peak eluting at a retention time of 13 minutes. Cotton, wool, and polyester showed accumulation of PCE over multiple dry cleaning cycles. Over multiple cycles, cotton had lower measured total amounts relative to polyester or wool. Cotton and polyester plateau at 2 and 3 cycles, respectively. Wool continued to increase steadily, having nearly two times the amount as polyester and nearly four times the amount of cotton after 6 dry cleaning cycles. These levels of PCE volatilize into ambient room air, regardless if they are wrapped in conventional dry cleaner plastic wrap.

The observation that silk does not contain residual PCE by our extraction method suggests either that the fabric does not absorb appreciable PCE during the cleaning process, or that silk may be less permeant to methanol. The later seems unlikely. Raw silk fibers produced by the *Bombyx mori* silkworm are comprised primarily of two proteins: an outer coating of sericin and fibroin (Taddei *et al.* 2007). The former has adherent properties and the latter is a fibrous protein primarily composed of the amino acids Gly, Ser, and Ala (Taddei *et al.* 2007). The approximately 50% Gly composition enables tight packing of silk fibers, while hydrogen bonding between the backbone and Ser side chains affords significant tensile strength (Taddei *et
al. 2007). This tight packing and sequestration of hydrophilic Ser residues in hydrogen bonding, coupled with the sericin coating might explain silk’s resistance to PCE absorption. In contrast, wool exhibits a heterogeneous protein composition leading to an overall amino acid sequence spanning the full range of charged, hydrophobic, and hydrophilic side chains, with the majority being the former two (Corfield et al. 1955). This amphoteric nature of wool combined with the arrangement of its fibers relative to silk explains, at least in part, why wool retains considerable residual PCE and continues to do so as the number of dry cleaning cycles increase (see also Kawauchi et al. 1989; Brodmann et al. 1975).

More work testing for PCE levels in other fabric types and other locations is clearly warranted. However, a key question arises from these initial data. Namely, do the measured PCE residues found in these fabric samples constitute risk to human health? PCE is classified as a class II carcinogen, but to have health effects the solvent must also be ingested or absorbed. One obvious route for transfer of clothing PCE residue to humans is absorption through the skin from fabric that makes skin contact. However, another would be PCE vapor emanating from fabric, particularly when it has been dry cleaned multiple times and is placed in a warm, closed environment such as the interior of an automobile on a sunny day. It is important to note that although the boiling point of liquid PCE is 120 °C, some evaporation from fabrics is observed even under ambient room temperature, with the amount extracted reduced by half after 7 days.

It is difficult to extrapolate actual human dose from either of these scenarios without additional data. However, we note that a pair of adult wool pants has approximately 30,000 cm$^2$ of material. Extrapolating from Table 7.1, such a clothing item could contain as much as 1300 µmol (160 mg) of PCE. Poet et al observed an average absorption of 167 ± 64 µmol PCE at a
rate of $0.0009 \pm 0.0003$ cm/h through the skin of human subjects following immersion of a human hand in 4 kg of soil containing approximately 1 mol PCE (Poet et al. 2002). This corresponds to absorption of 0.017% of the available PCE (Poet et al. 2002). If a similar percentage of the PCE in the clothing article discussed above was absorbed through skin, and if 120 such items were worn over a one-year period the person wearing these garments would have received a dose of PCE corresponding to 27 μmol (approximately 3 mg). However, it is important to note that hand skin tissue was not exposed to the entire 1 mol of PCE in (Poet et al. 2002) and that the efficiency of soil/skin partitioning vs. fabric/skin partitioning likely differs considerably (hand derma is much thicker), leading to an underestimation of the above calculated “dose” from a wool garment. Also, PCE prefers partitioning to fat tissue which is largely absent from the hand but prevalent elsewhere in torso.

Alternatively, if 10 such items remained in a sealed closet area, then 6.5 mmol of PCE would presumably be released into the closet air supply over a period of 7 days. For an average-sized closet of 3.4 m$^3$, this corresponds to a total exposure of 2.0 mmol/m$^3$ (0.24 g/m$^3$) or 50.0 ppm (EPA 1994). By similar logic, four wool sweaters subjected to six dry cleaning cycles placed in a warm 3.4 m$^3$ car interior would result in 5.13 mmol/m$^3$ (0.62 g/m$^3$) or approximately 126 ppm, assuming all residual PCE is vaporized.

The question of whether such dosages are carcinogenic is difficult to answer given the highly inconclusive and conflicting nature of only a handful of rat carcinogenicity studies reported in the literature. Of nine such studies conducted, three focused on PCE administration by inhalation and six by oral gavage (Ishmael and Dugard 2006). Two of the inhalation studies reported the incidence of mononuclear cell leukemia (MCL) in Fisher 344 (F344) inbred rats and
increases in hepatocellular adenomas and/or carcinomas in mice. A National Toxicology Program Technical Report (1986) indicated a 18% increase above control in male rats exhibiting MCL and a 24% increase in females following a PCE dose of 200 ppm (8.2 μmol/cm³, 8200 mmol/m³) for 6 h/d (EPA 1994; NTP 1986). Nagano et al report a 33% increase in MCL incidence for male F344 rats and a 12% increase for female F344 rats receiving this same dose of PCE (Nagano et. al 1998). However, in both cases and the results were deemed unsuitable for extrapolation to humans (Ishmael and Dugard 2006; NTP 1986; Nagano et. al 1998). We suggest definitive conclusion can only be made when skin absorption parameters for these levels of PCE that we detect are rigorously defined.

For these reasons, we suggest further detailed quantification of PCE residues is warranted. We also suggest that studies human exposure to these residues be carefully evaluated, particularly PCE absorption through the skin, post-absorption bioavailability, and carcinogenic vs. toxic levels of absorbed vs. inhaled PCE.

7.1.5. Acknowledgements

We thank the Dantzler family of Manassas, VA for assistance with dry cleaning samples; Dr. Ron Davis, Jr. for instrumental guidance and support; Mrs. Doreen Curtin, Science Department Chair at Bishop O'Connell High School for assistance and Ms. Rebecca Dzubow (MPH, MEM) of the U.S. E.P.A. for helpful discussions. This work was supported by the Georgetown University Department of Chemistry, and a grant–in–aid from the Georgetown University Graduate School.
7.2. Natural Functions of Drug Resistance Associated Membrane Proteins.


Study of drug resistance began in the late 1940’s, and recognition that altered membrane transport of drug was often related to cellular drug resistance followed approximately 20 years later. Identification and isolation of specific membrane proteins that influence this altered drug transport began in the 1970’s and is a major ongoing endeavor to this day. In this article, I refer to such proteins as “drug resistance associated membrane proteins”, or “DRAMPs”. In over 40 years, dozens of books and tens of thousands of research articles have asked how drug resistance is mediated by DRAMPs. By comparison, relatively few studies have probed the normal physiological function of these proteins. In many cases, deletion of the gene encoding a DRAMP is not lethal, showing that the function of the protein is non-essential, but in some cases (e.g. PfCRT protein involved in antimalarial drug resistance) deletion is not possible, suggesting an essential function. For some DRAMPs a clear role in specific cell biological processes has been established (e.g. Jin *et al*. 2002; Ishikawa *et al*. 1997; Quazi *et al*. 2013; Baugh *et al*. 2012), but in most cases we are no closer to a detailed molecular definition of the physiologic function of DRAMPs than we were when these proteins were first discovered. When they are involved in drug resistance, DRAMPs are often either mutated or overexpressed, and in some cases both. Quantitative comparison between wild type and mutant isoforms of DRAMPs, or between
normal and higher levels, is often quite difficult for a variety of technical reasons, and this has probably slowed elucidation of their physiologic function.

The vast majority of genetic, biochemical, biophysical, and cell biological studies with DRAMPs have emphasized their putative interactions with drugs, and dozens of review articles summarize decades of such work (e.g. Saidijam et al. 2006; Blair et al. 2009; Bay et al. 2008; Damme et al. 2011). The known array of DRAMPs is now dizzying, with hundreds of proteins now organized into five families (ABC, MATE, MFS, SMR, RND), as described elsewhere (Alvarez-Ortega et al. 2013). Members of each family can be found in multiple phyla, but sequence conservation across phyla is typically quite low. Individual members of these families have been implicated in anticancer, antibacterial, antifungal, and antiparasitic drug resistance phenomena. Other papers in this volume describe specific proteins in detail. Collectively, these data related to DRAMP structure and function and their roles in drug resistance are exceedingly rich and illuminating, and encompass a remarkable diversity of function. It is therefore a challenge (and perhaps less effective) to view them as a single class of proteins, since their only common thread is participation in drug resistance phenomena, which are biologically and chemically quite diverse.

To add additional complexity, there are four possible routes to cellular drug resistance: 1) catabolism of the drug, 2) mutation and/or altered expression of the drug target, 3) switching off a relevant metabolic pathway or 4) altered cellular transport of the drug. All operate simply to reduce the efficiency of interaction between the drug and its molecular target, and membrane proteins involved in drug resistance phenomena could in theory influence any of the four routes. To date, most DRAMPs studied in depth have been associated with altered drug transport
phenomena that act to promote lower drug-drug target association. The most famous of these is human MDR1 protein (P-glycoprotein), which mediates decreased accumulation of anticancer drugs in tumor cells (Quazi et al. 2013; Roepe et al. 1996). Early on it was appreciated that altered drug transport induced by overexpression of huMDR1 could in theory be direct or indirect (10), meaning huMDR1 could mediate direct translocation of drugs back out of the cell to reduce net accumulation, or indirectly influence accumulation of drugs through physical chemical effects, such as changes in membrane potential that would then effect passive influx of some drugs (Wadkins and Roepe 1997). Decades later, multiple examples of both direct and indirect phenomena can be found for various examples of drug resistance mediated by DRAMPs. Another question raised early on was whether degrees of resistance or patterns of drug “cross resistance” were mediated solely by huMDR1 and other DRAMPs. In addressing this question, of note is the fact that many early models of drug resistant cells were created by incremental exposure to increasing concentrations of a single drug (Biedler et al. 1970), protocols that induce a variety of “epi-phenomena” that are now known to add to drug resistance, along with DRAMPs. For example, it is now appreciated that the 100’s to 1000’s-fold levels of drug resistance observed in early drug selected tumor cell models are clearly not due to huMDR1 overexpression alone. A crucial concept that emerged from this period is that altered cell signaling related to programmed cell death is likely more relevant for high levels of drug resistance in many cancers, relative to the contribution made by ABC DRMPs (Lowe et al. 1993; Borst 2012).

A central, remaining question for most of these proteins is what functions do DRAMPs have in the absence of drugs to which resistance has been selected? Even if not essential in
many cases, clearly these functions are important since the proteins are chromosomally encoded, are found in all phyla, and have been evolving for millions of years. Even in the absence of man-made drug pressure for isolated microbiomes, bacteria have been discovered with a wide-range of resistance phenotypes suggesting that one normal physiologic function is conferring resistance to commensal defensins or other excreted natural antibiotics (Bhullar et al. 2012). Most drug resistance researchers believe that molecular definition of these “normal” or “physiologic” functions will better illuminate the molecular mechanism of how the membrane proteins confer drug resistance. Presumably, mutations or increased expression seen for these proteins in drug selected cells confers drug resistance phenomena because the normal function is “hijacked” or altered in such a way as to reduce interaction between drug and drug target. But beyond the example of “naturally resistant” bacteria (Bhullar et al. 2012), what are those normal functions?

DRAMPs in bacteria have identified natural functions that include: spermidine degradation, pH homeostasis, alkali tolerance, as well as removal of naturally-produced antibiotics, toxic fatty acids, bile salts, homoserine lactones, and aromatic hydrocarbons (Neyfakh 1997; Krulwich et al. 2005; Fernandez et al. 2012). For huMDR1 and its many relatives in the ABC transporter superfamily, the most illuminating early experiments came from gene knockout experiments with mice (Smit et al. 1993). Here, Borst and colleagues showed a distinct tissue/cellular phenotype associated with deletion of murine orthologues of huMDR1, namely, altered traffic of phospholipids. This suggests that the normal function of huMDR1 may include maintenance of cell membrane lipid disposition, or direct transport of phospholipids and related molecules. These hypotheses were supported in several follow-up reports (e.g. van Helvoort et al. 1996; Raggers et al. 2000; Romsicki et al. 2001). Work in this area remains
ongoing, and the only universally accepted conclusion at this point seems to be that there is a wider-than-expected diversity of natural substrates for ABC transporters involved in drug resistance phenomena.

Another important class of DRAMPs is comprised of those involved in anti-parasitic drug resistance. Although they are not the only mechanism that confers resistance, transporters have been implicated in drug resistance for the microorganisms that cause schistosomiasis (Kasinathan et al. 2012), leishmaniasis (Ait-Oudhia et al. 2011), sleeping sickness (Wilkinson and Kelly 2009), malaria (Roepe 2011), and other parasitic diseases. Perhaps the most heavily studied to data are malaria DRAMPs. These include orthologues of huMDR1 and huMRP that are found in multiple species of malarial parasites, including *P. falciparum* and *P. vivax*. Early on (Foote et al. 1990; Wilson et al. 1989), PfMDR1 was thought to be the major contributor to the most widespread form of antimalarial drug resistance (chloroquine [CQ] resistance, [CQR]), however, mutations in a novel DRAMP with no known orthologues in other eukaryotes, called PfCRT, was subsequently found to be responsible for a much larger portion of the shift in CQ cytostatic activity (CQ IC\(_{50}\)) observed for CQR *P. falciparum* (Fidock et al. 2000). Multiple mutations in the *pfcrt* gene are required for CQR, and these confer patterns of amino acid substitutions in the encoded PfCRT protein. The patterns reveal the geographic origin of the CQR strain or isolate and also suggest different cross-resistance patterns to related drugs (Summers et al. 2012). There are now at least 17 distinct isoforms of PfCRT known to exist, with each isoform selected by different drug-use histories in the respective geographic origin (Baro et al. 2013). For at least a decade, it has been widely suspected that these PfCRT
mutations may be all that is necessary to confer resistance to multiple quinoline antimalarial drugs, and perhaps other compounds.

Recently however, in set of observations oddly reminiscent of the now-accepted concept that huMDR1 protein overexpression is only part of the explanation for tumor multidrug resistance, we now know that PfCRT mutations are only part of the story for CQR. From cancer cells to malarial parasites, altered signaling related to drug induced programmed cell death also appears necessary for high-level drug resistance (e.g. Lowe et al. 1993; Gaviria et al. 2013). Using progeny of an available CQR x CQS parasite genetic cross, a very recent paper shows that the genetics of “cytostatic CQR” (resistance to cytostatic, or growth inhibitory, effects of the drug) are distinct from those of cytocidal CQR (resistance to cytocidal, or parasite kill, effects of the drug) (Gaviria et al. 2013). That is, similar to high levels of tumor cell multidrug resistance, and other examples of bacterial, fungal, and parasite drug resistance, it now appears likely that a DRAMP (PfCRT) is responsible for one key layer to antimalarial drug resistance, with altered signaling related to parasite cell death adding to the phenotype upon acquisition of high levels of cytocidal drug resistance (Gaviria et al. 2013; Sinai and Roepe 2012; Roepe 2014).

Returning to the question at hand, what does this tell us about studies that probe DRAMP physiologic function? An important lesson is that studies attempting to define physiologic function using drug pressured or drug selected cell lines can confuse analysis due to the presence of unforeseen epi-phenomena, such as altered metabolism and signal transduction related to cell death pathways. Similar to defining the precise contribution of the DRAMP to drug resistance, it can be difficult in these drug selected systems to unambiguously define physiologic function because other epi-phenomena are present. Direct gene knockout experiments can in some cases
be more informative, except of course in examples where the DRAMP is essential for cell viability (Waller et al. 2003). The ultimate goal for molecular definition of DRAMP function is purification and reconstitution into a lipid bilayer or proteoliposome preparation, followed by direct molecular assays. A number of such experiments have been done for huMDR1 and other eukaryotic ABC transporters (e.g. Sharom et al. 1993; Ambudkar et al. 1998; Howard and Roepe 2003), bacterial DRAMPs (e.g. Yershalmi et al. 1995) and even malarial DRAMPs (Paguio et al. 2009). Some of these studies have been used to query possible natural substrates, but many more such experiments are needed.

Even once the protein is purified and reconstituted, functional assays for DRAMPs can be quite difficult, in part because the suspected transporter substrates are often hydrophobic molecules or lipophilic metabolites. Also, some DRAMPs may not even be transporters, but signaling receptors involved in cell death that require yet unknown co-factors. In the case of hydrophobic substrates, routine transport assays that separate proteoliposomes from substrate at various time points via centrifugation or filtration have very high background due to the substrate adhering to lipid, filters, etc. Another approach that shows promise, at least for known substrates, is to use a fluorometric substrate analogue wherein a fluorescent signal can be used to distinguish drug on one side of a membrane vs. another. For example, this has recently been used to monitor CQ transport by PfCRT (Paguio et al. 2009). This analysis reveals features of drug transport by PfCRT that are not possible to monitor with other approaches (Roepe 2011).

Yet, similar to most other DRAMPs, for PfCRT and PfMDR1, we still have no unequivocal molecularly defined function for the protein in normal cell physiology. For PfCRT cell biological clues abound, and in particular include some evidence for a role in organellar
volume regulation, ionic equilibria, hemoglobin metabolism, and perhaps glutathione traffic
(Gligorijevic et al. 2006b; Bennet et al. 2004a; Lewis et al. 2014; Patzewitz et al. 2013). Further
testing these hypotheses, with purified protein in reconstituted systems such as proteoliposomes
and bilayers, remains an important goal as is the case for all DRAMPs. The papers in this
volume offer invaluable guidance in this ongoing endeavor.

7.2.1. Acknowledgments

Work in the Roepe laboratory mentioned here has been supported by the NIH, the
Burroughs Wellcome Fund, DoD, and the Luce Foundation.
7.3. Determination of the Cytostatic and Cytocidal Activities of Antimalarial Compounds and their Combination Interactions


7.3.1 Introduction

The rise of antimalarial multidrug resistance and the failure of current antimalarial therapies necessitate the identification of new drugs and drug combination therapies. Notwithstanding difficulties inherent in live malaria parasite cell culture that typically requires coculture with human erythrocytes, this protocol describes simple and inexpensive assays that can be used to investigate the activity of potential antimalarials. The use of the fluorescent reporter SYBR Green I takes advantage of the lack of nuclei in erythrocytes, and minimizes background due to its increase in fluorescence intensity upon intercalation with double stranded DNA (Bennet et al. 2004). This improves upon earlier assays that required expensive reagents such as radioactive-labeled hypoxanthine (Desjardins et al. 1979), monoclonal antibodies (Druilhe et al. 2001), or extensive washing to prevent quenching due to hemoglobin (Quashie et al. 2006).

Assays can be tailored to study cytostatic growth inhibition (Bennet et al. 2004), higher-dose cytocidal cell kill (Paguio et al. 2011), as well as the effect of drug combinations following methods described by Chou and Talalay (Chou and Talalay 1984; Suberu et al. 2013). The differences between the cytostatic and cytocidal assays include constant vs. bolus dosing, altering drug concentrations and adding washing steps. Where necessary, quantification of cytocidal potency (e.g. determination of LD$_{50}$) can be further tested using a long-term limiting
dilution assay adapted from a recently described rate of cell-kill assay (Sanz et al. 2012). Adapting any of these assays for drug combination analysis requires the generation of serial dilutions at multiple fixed drug ratios near the pharmacologically-relevant doses for each drug in the combination.

**Materials**

*Plasmodium falciparum* culture at 2% hematocrit (volume packed RBC/volume media)

Complete Media

Type O* human serum (off-the-clot, heat-inactivated)

Fresh O* human whole blood, washed to isolate the erythrocytes, and stored in Incomplete Media.

10% Giemsa Dye

Drug stocks (in DMSO, deionized water, or 50% ethanol)

96-well plate, clear well bottom with opaque well sides to prevent fluorescence interference

10,000x SYBR Green I stock from the vendor (Invitrogen), or synthesized (see Bennett et al., 2004)

5% CO₂, 5% O₂, and 90% N₂ gas mix

*Specialized Equipment*

Air-tight chamber

37°C Incubator

Eppendorf 5804 centrifuge with A-2-DWP rotor

Spectra Max Gemini™ EM Microplate Reader, or similar bottom-read fluorescence plate-reader

Microsoft Excel, Sigma Plot, or other data-analysis program that allows non-linear curve fitting
7.3.2. Cytostatic (IC$_{50}$) Growth-Inhibition Assay- (adapted from Bennet et al. 2004)

This protocol is used to assess the ability of a compound to slow the growth of bulk intraerythrocytic *P. falciparum* culture. Where possible, this should be measured over the course of more than 1 parasite life cycle (more than 48 hours). The compound, in various concentrations, is continuously incubated with low parasitemia culture, and growth is assessed relative to a no-drug control through use of a fluorescent DNA-intercalating dye, SYBR Green I. The 50% growth inhibitory concentration (IC$_{50}$) is determined through non-linear curve fitting of the data. The use of multiwell-plates and a fluorescence plate reader allows for high-throughput screening, and the assay has been successfully adapted to 384 and even 1536 – well plate formats.

1. Using a Giemsa smear, calculate *P. falciparum* culture parasitemia and adjust culture to 4% hematocrit and 1% parasitemia in culture media (% parasitemia is the percentage of red blood cells that are infected, hematocrit is the volume percent of packed red blood cells).

2. Generate drug solutions at 2x the desired target concentrations in complete media.

   Aliquot 100 µL of each concentration into each of three wells in a 96-well plate. We recommend 3 wells for each drug concentration such that each assay is done in triplicate, and we recommend 3 independent assays (9 determinations in total) for each drug to compute reliable IC$_{50}$. Include no-drug controls for each strain of *P. falciparum* that is being analyzed, as shown schematically in Table 7.2.
Table 7.2. Diagram of Plate for Cytostatic Assay.

<table>
<thead>
<tr>
<th></th>
<th>HB3 (CQS)</th>
<th>Dd2 (CQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 µM CQ</td>
<td>10 µM CQ</td>
</tr>
<tr>
<td>2</td>
<td>No drug HB3</td>
<td>No drug Dd2</td>
</tr>
<tr>
<td>3</td>
<td>10 µM CQ</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
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<td>12</td>
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</table>

3. Add 100 µL of the 4% hematocrit/1% parasitemia culture to each of the assigned wells, producing a sample at 1x drug concentration and 2% hematocrit/1% parasitemia, mixing well to ensure the RBCs are fully suspended in the media.

4. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.

5. Incubate at 37°C for 72 hours, flushing the chamber once a day with new gas.

6. Prepare a parasite-standard curve by counting a Giemsa smear and setting up small cultures at 2% hematocrit and a range of predetermined % parasitemias and add to plate wells in triplicate. Include a 0% parasitemia (RBCs in culture media) to account for background fluorescence, as shown in Table 7.3.
Table 7.3. Diagram of Cytostatic Assay Plate with Standard Curve Cultures.

<table>
<thead>
<tr>
<th></th>
<th>HB3 (CQS)</th>
<th>Dd2 (CQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 µM CQ</td>
<td>No drug HB3</td>
</tr>
<tr>
<td>B</td>
<td>500 nM CQ</td>
<td>1% p HB3</td>
</tr>
<tr>
<td>C</td>
<td>250 nM CQ</td>
<td>2% p HB3</td>
</tr>
<tr>
<td>D</td>
<td>100 nM CQ</td>
<td>4% p HB3</td>
</tr>
<tr>
<td>E</td>
<td>50 nM CQ</td>
<td>6% p HB3</td>
</tr>
<tr>
<td>F</td>
<td>10 nM CQ</td>
<td>8% p HB3</td>
</tr>
<tr>
<td>G</td>
<td>5 nM CQ</td>
<td>10% p HB3</td>
</tr>
<tr>
<td>H</td>
<td>1 nM CQ</td>
<td>RBCs</td>
</tr>
</tbody>
</table>

7. Prepare a solution of 50x SYBR Green I in culture media. Add 50 µL of the SYBR Green I solution to each well (final concentration 10x), mixing well to fully suspend the RBCs, while also protecting from direct light.

8. Place the plate back into the air-tight chamber, gas, and store at 37°C for 1 hour, protecting from light.

9. Measure fluorescence for each well ($\lambda_{ex}$ 490 nm; $\lambda_{em}$ 538 nm with 530 nm band-pass and 538 nm long-pass filters) using the Spectra Max Gemini™ EM Microplate Reader, or similar bottom-read fluorescence plate-reader, while also setting the internal temperature to 37°C (note some plate readers require 5 – 10 min pre – equilibration to correct temperature).

10. Transfer the raw fluorescence data to Microsoft Excel or a similar data analysis program.

11. Calculate fluorescence background by averaging the three values for the RBC-only wells, and subtract this value from values for all other wells on the plate.
12. Generate a standard curve for each parasite strain by plotting the average fluorescence vs. predetermined parasitemia, and fit to a linear equation.

13. Average the fluorescence for each sample (and the no-drug control), and use the respective standard curve to convert these values to % parasitemia.

14. Divide the % parasitemia for each drug treated sample by the % parasitemia of the no-drug control and plot % growth vs. [drug].

15. These growth curves can be fit to a sigmoidal function, which is then used to calculate the drug concentration that would yield 50% growth. This is the IC\textsubscript{50}.

7.3.3. Cytocidal (LD\textsubscript{50}) Cell-Kill Assay- (Paguio \textit{et al} 2011).

This protocol adapts similar methods to determine the concentration of a bolus dose of drug that kills 50% of parasites (e.g. calculates the LD\textsubscript{50}). Choice of the length of time for the bolus dose can be varied to reflect drug lifetime in media or plasma, as well as to probe any parasite stage specificity for the activity of the drug. The parasites are incubated with the compound for less than 1 life-cycle (typically 6 hours as a starting point, see Paguio \textit{et al.}, 2011) and then the drug is washed away. The surviving parasites are allowed to grow through one complete life cycle before staining with the same SYBR Green I dye. Comparison to no-drug control allows for determination of the 50% lethal dose (LD\textsubscript{50}). The LD\textsubscript{50} that is calculated often (but not always) depends on the bolus incubation time and can be parasite stage – specific (Paguio \textit{et al.}, 2011). Also, additional control experiments that calculate LD\textsubscript{50} at different endpoints should be done for drugs that might potentially induce extended quiescence phenomena along with death, if distinction between these is desired.
1. Using a Giemsa smear, count the *P. falciparum* and set up a culture at 4% hematocrit and 2% parasitemia in culture media.

2. As in the cytostatic assay, generate a series of drug concentrations at 2x the desired target concentration in complete media and aliquot 100 µL into each of three wells in the 96-well plate, as well as no-drug controls for each strain.

3. Add 100 µL of the 4% hematocrit/2% parasitemia culture to each of the assigned wells, producing a sample at 1x drug concentration and 2% hematocrit/1% parasitemia, mixing well to ensure the RBCs are suspended in the media.

4. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.

5. Incubate at 37°C for 6 hours.

6. Wash the plate by spinning in an Eppendorf 5804 centrifuge with A-2-DWP rotor at 700g for 3 min, removing the supernatant from each well carefully to avoid disturbing the pellet (using a multichannel pipette), and resuspending in 200 µL of culture media. Repeat three times, for a total of 3 washes.

7. Return the plate to the air-tight chamber, flush with gas, and incubate at 37°C for 48 hours, flushing the chamber with new gas each day.

8. Repeat steps 6-15 from the cytostatic assay protocol. Plot as % survival vs. drug dose to compute LD<sub>50</sub>. 

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7.3.4. Limiting Serial-Dilution Assay- Adapted from (Sanz et al. 2012).

Warning: this assay requires extensive parasite culturing and 30 days to complete. It is more expensive and (much) more tedious than the above two assays.

To rigorously test conclusions from the cytocidal assay described above, this assay determines the cell-kill ability of a compound through a limiting dilution method. Comparison between parasite-free controls and cultures diluted down to (in theory) 1 parasite per well can be used to back-calculate the number of viable parasites that survived a bolus drug dose. Plotting this survival vs. concentration of bolus drug treatment allows calculation of the LD$_{50}$.

1. Using a Giemsa smear, count the *P. falciparum* and set up a culture at 4% hematocrit and 2% parasitemia in culture media

2. As in the cytostatic and Paguio cytocidal assays, generate a series of drug concentrations at 2x the desired target concentration in complete media and aliquot 100 µL into each well in the 96-well plate.

3. Add parasites as in the above two assays, to generate final 2% hematocrit / 0.5 % parasitemia. Note that % parasitemia can be adjusted, but in general a lower initial parasitemia is used because these plates will develop for a much longer time (30 days) and culture will “crash” if parasitemia becomes too high.

4. Place the plate in an air-tight incubator chamber and flush with a 5% CO$_2$, 5% O$_2$, and 90% N$_2$ gaseous mix for 3 minutes to ensure a proper atmosphere.

5. Incubate at 37°C for 6 hours (6 hours bolus drug treatment; again, time can be varied depending on what concepts are being explored, see Paguio *et al.*, 2011).
6. While the parasites are incubating with the drug, prepare 2% hematocrit RBCs in complete media (40 mL per strain being analyzed). This large amount of culture will be needed for making serial dilutions for the limiting dilution approach.

7. Wash the drug treatment plate by spinning in an Eppendorf 5804 centrifuge with A-2-DWP rotor at 700g for 3 min, removing the supernatant from each well carefully (using a multichannel pipette), and resuspending in 200 μL of culture media. Repeat two more times, for a total of 3 washes. This step should remove drug; note that some antimalarial drugs (e.g. artemether, artemisinin and artesunate) covalently modify their target(s) and cannot be completely washed away. This adds some level of additional complexity to interpretation of the results.

8. Dilute 40 μL of the drug treated wells into 160 μL of fresh RBCs, serially, to reduce parasite load per well from 400,000 (starting parasitemia) to 16,000, see Figure 7.9.

9. Dilute 35 μL of the 16,000 parasite / well culture into 165 μL fresh RBCs in the first column of a new plates, reducing the parasite load per well to 1,400, see Figure 7.9.

10. Serially-dilute this mixture by diluting 100 μL of this mixture into 100 μL of fresh RBCs for the well in the next column, continue for each of the remaining columns. Discard 100 μL from the last (11th) column, reserve the 12th column for a background measurement (RBCs only; no parasites), see Figure 7.9. Each plate is a single assay; in a standard 96 well plate the additional rows can be used for replicates, additional strains, or additional culture treated with different bolus drug dose. Note that to calculate LD₅₀ 6 – 10 different drug doses will be needed. As for the Bennett and Paguio assays, we represent 3 independent assays, each done in triplicate, for 9 determinations in total. If each drug
dose in analyzed in triplicate, the assay requires 4 plates per strain per drug (e.g. 4 plates are required to calculate CQ LD$_{50}$ for one strain of *P. falciparum*).

**Figure 7.9.** Plating of the Limiting Dilutions. 400,000 drug-treated (and washed) parasites are diluted to achieve a final concentration of 1 parasite per well utilizing the original plate (Drug Treatment Plate) as well as a second plate (Parasite Dilution Plate).
11. The number of parasites per well should (in theory) now be as shown in Table 7.4.

**Table 7.4. Hypothetical Number of Parasites in Each Dilution of the Limiting Dilution Assay.**

<table>
<thead>
<tr>
<th>Column</th>
<th># of Parasites per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,400</td>
</tr>
<tr>
<td>2</td>
<td>700</td>
</tr>
<tr>
<td>3</td>
<td>350</td>
</tr>
<tr>
<td>4</td>
<td>175</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

12. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.

13. Incubate at 37°C for 4 weeks, with maintenance (see below), flushing the chamber with balanced gas every day.

14. After 1 week of incubation, make a new batch of 2% hematocrit RBCs in complete media, and add 100 μL to each well to feed the culture. Put back in the chamber, gas, and return to 37°C incubator.
15. On week 3, centrifuge the plates to pellet the cells, and replace 50 µL of the supernatant with 50 µL of fresh culture media. Put back in the chamber, gas, and return to 37°C incubator.

16. Develop the plates as described in steps 7-10 of the cytostatic assay protocol, but using a 10x SYBR Green I solution instead of 50x.

17. Average the values for each drug concentration at each dilution factor.

18. Count the wells that show fluorescence signal above the RBC alone background (the 12th column).

19. Back-calculate the number of starting parasites that have survived (P) using the following formula, where X is the dilution factor of the culture (2 if done as described above), and n is the number of wells that showed parasite growth:

\[(1) \quad P = X^{n-1}\]

20. Plot the calculated number of parasites that have survived vs. the drug concentration. It can be helpful to display the concentration axis in log scale.

21. Fit the data to a sigmoidal curve and determine the LD\(_{50}\) by identifying the concentration that produces 50% effect, relative to complete parasite kill.
7.3.5. Modifying the Cytostatic and Cytocidal Assays for Assessing Synergy of Drug Combinations- Adapted from (Chou and Talalay 1984; Suberu et al. 2013)

In order to investigate potential drug combination therapies, the cytostatic and cytocidal assays can be adapted to standard methods for defining whether drug interactions are synergistic, additive, or antagonistic. Typically this starts with two different drugs mixed at a defined ratio, but in theory drug combinations of three or higher can also be analyzed in an iterative approach. Using serial dilutions of a fixed ratio of the drugs near their individual pharmacologically-relevant doses, a growth or survival curve is generated vs. the concentration of each compound and the fractional inhibitory concentration / fractional lethal dose of each compound is determined. In the “1 point” Chou – Talalay approach, summation of these FIC values allows for determination of the combination index and designation of the type of interaction.

1. Both the cytostatic and cytocidal assays can be adapted for the identification of synergy, additivity, and antagonism between two drugs in a fixed-ratio combination.
2. In order to analyze a combination, the individual (monotherapy) activity of each compound must first be determined as described above.
3. Make a stock solution of each drug at 16 x IC\textsubscript{50} (or LD\textsubscript{50}) in complete culture media. Mix these solutions to generate a fixed volume ratio (each drug now at 8 x). The ratio may be expressed in terms of absolute concentration (molar ratio of each drug) or in terms of relative IC\textsubscript{50} / LD\textsubscript{50} (i.e. we often express a ratio that contains IC\textsubscript{50} for drug A and IC\textsubscript{50} for drug B as a “1:1 IC\textsubscript{50}” ratio.
4. Serially dilute the mixture two-fold across a 96 well plate, 7 times to yield a set of 8 samples.
5. Add 100 µL of each dilution to 96-well plate in triplicate, also include no-drug control wells.

6. Add 4% hematocrit culture to the plate (suggested 2% parasitemia for cytocidal, 1% parasitemia for cytostatic), 100 µL in each well, including the no-drug control.

7. Follow steps 4-15 of the cytostatic assay protocol. However, when plotting the growth/survival data, two are required, and each must be plotted versus the absolute concentration of either drug in the mixture. The curve fits then generate not an IC$_{50}$ or LD$_{50}$, but what is often termed the “pseudo IC$_{50}$” or “pseudo LD$_{50}$”. Pseudo IC$_{50}$ and LD$_{50}$ are often specific for the drug ratio that is being examined. Different drug ratios can produce different pseudo IC$_{50}$ or LD$_{50}$, revealing different drug interactions (i.e. in theory a drug combination can show synergy at one ratio but antagonism at another).

8. Convert the pseudo IC$_{50}$/ pseudo LD$_{50}$ into a fractional inhibitory concentration (FIC)/fractional lethal dose (FLD) for each compound using the following equations:

\[
(2) \quad FIC = \frac{\text{pseudoIC}_{50}}{\text{monotherapy IC}_{50}}
\]

\[
(3) \quad FLD = \frac{\text{pseudo LD}_{50}}{\text{monotherapy LD}_{50}}
\]

9. Calculate the combination index (CI) by summing the two FIC/FLD values for the two compounds:

\[
(4) \quad CI_{AB} = FIC_A + FIC_B
\]

\[
(5) \quad CI_{AB} = FLD_A + FLD_B
\]
10. Use the CI to assign synergy (CI ≤ 1), additivity (1 < CI ≤ 2), or antagonism (CI > 2) (Bhattacharya et al. 2009; Matthews et al. 2013). Note that the choice of cutoff values for determining additivity vs. antagonism can be somewhat arbitrary in the literature.

7.3.6. Reagents and Solutions

Complete Media- store at 4°C for up to one week

Incomplete Media

10% type O+ human serum

25 mM HEPES

20 μg/L gentamicin

Adjust pH to 7.4 when at 37°C

Incomplete Media- store at 4°C for up to one month

RPMI 1640

23 mM NaHCO₃

11 mM glucose

0.75 mM hypoxanthine

7.3.7. Commentary

Background Information

The rise of multidrug resistance in P. falciparum malaria has renewed interest in the identification of novel candidate drugs and novel drug combination therapies. In order to screen for new drugs, various in vitro assays have been developed that utilize radioactive hypoxanthine
incorporation (Desjardins et al. 1979), ELISA detection of parasite lactate dehydrogenase (Druilhe et al. 2001), or various DNA-intercalating dyes which take advantage of the lack of nuclei in red blood cells (RBCs) (e.g. Bennet et al. 2004; Quashie et al. 2006). In order to simplify these growth-inhibition assays, and to avoid the cost of radioactive-labeled compounds, lysing and extensive washing of cells, as well as dyes that require removal of hemoglobin or the use of UV illumination, 10 years ago we developed an assay that used SYBR Green I added directly to the infected RBCs (no washing steps required), with measurement via a fluorescence-capable well-plate spectrophotometer (Bennet et al. 2004). The combination of ease of use and the use of multiwell plates allows for the rapid and much less expensive screening of multiple compounds and multiple strains at once.

Recent molecular studies of drug resistance phenomena have generated considerable interest in more rapidly defining the ability of antimalarial compounds to kill Plasmodium parasites, not merely inhibit their growth. The classical mode-of-action of chloroquine that is typically invoked involves inhibiting the crystallization of toxic free heme that is released upon catabolism of host RBC hemoglobin by the intraerythrocytic parasite. However, a number of studies show that this activity does not correlate with the ability of the drug to kill parasites (Gligorijevic et al. 2008; Cabrera et al. 2009a; Paguio et al. 2011; Gaviria et al. 2013; Gorka et al. 2013a; Gorka et al. 2013b). In order to fully define the activity of an antimalarial drug, quantification of both cytostatic and cytocidal activity is required, leading recently to the development of newer assays that probe cytocidal potency (Paguio et al. 2011; Hasenkamp et al. 2013), or the rate at which a set concentration of a drug will kill parasites in vitro (Sanz et al. 2012). Adaptations of the SYBR Green I assay have been used to probe the differences between
cytostatic (lower dose, constant long-term drug exposure growth inhibition) and cytocidal (higher dose, short-term exposure cell kill) activities of common antimalarials and the cellular biochemistry that may be related to the resistance to either activity (Paguio et al. 2011; Gaviria et al. 2013).

Since the advent of artemisinin combination therapies (ACTs), it has become necessary to more thoroughly investigate drug combinations in vitro. Much of the work in developing drug combinations is (quite justly) focused on defining complementary pharmacokinetics and clinical efficacy, but it is also imperative to understand possible interactions the two compounds have within the parasite. These can be essential in analyzing potential cross-resistance for drug combinations (Burrows et al. 2013). Also, thorough investigation can in theory lead to identification of combinations where each compound selects against resistance to the other (Lukens et al. 2014). Determination of the type of interaction between potential combinations, (synergistic versus additive versus antagonistic), is a key layer to in vitro screening. Mathematical derivations performed by Chou and Talalay years ago are equally applicable to malaria drug screening. These have lead to hybrid techniques that entail assigning interactions based on fixed-ratio combinations (Chou and Talalay 1984). The Chou Talalay Method allows for investigation of possible synergy at a minimum of one combination ratio, as opposed to the generation of a full isobologram, which requires analysis of at several ratios (Berenbaum 1978).

Critical Parameters

In order to obtain a full growth/survival curve, it may be necessary to run a 3 or 4 point titration across several orders of magnitude in order to determine the appropriate range for a complete 8-point assay. For reliable quantification, typically the 8-point range will need to cover
about 3 orders of magnitude in order to cover the full parasite response of 100% growth/survival to 0% growth/survival regions. In order to account for error intrinsic to the use of parasite cell culture, the assay should be run independently at least 3 times with 3 different culture preparations. The triplicate wells run in a single assay account only for in-assay variations (SYBR Green loading, plate-reader instrumental error, aliquoting of culture into the plate, etc.).

Troubleshooting

In order to obtain reliable curve-fits, every attempt should be made to include concentrations that fall between 0 and 100% growth/survival. If the initial 8-point assay does not encompass the full range of activity, it should be run again since the fit may not accurately quantify IC$_{50}$ or LD$_{50}$. The largest source of inconsistency in these assays is in the growth of the culture. In order to maximize signal-to-noise in the fluorescence readings, additional no-drug controls can be included on a separate plate and developed with SYBR Green at different times (e.g. 48 hrs vs. 54 hrs, etc). If the dynamic range between the background RBC fluorescence and the culture fluorescence is not at least 4-fold, it may be necessary to let the plates incubate for up to 24 hours longer in order to allow for additional growth and higher signal to noise. Alternatively, starting parasitemia can be adjusted to fine tune dynamic range. We find it useful to include well characterized reference strains (e.g. CQS HB3 and CQR Dd2) and a well characterized reference drug (e.g. CQ) in all our assays as an “internal control” for these and other variables. If IC$_{50}$ or LD$_{50}$ for the well characterized example is not found, trouble shooting is called for!
Anticipated Results

Typically, the growth/survival curve will show plateau at 100 and 0%, respectively, with the slope of the curves requiring a span of about 2 orders of magnitude in drug concentration. However, it is possible for these plateaus to occur slightly above 100%, or to not fully reach 0%. These deviations from theory can be due to culture conditions, or to more complex drug pharmacology / drug interactions than space permits here.

Time Considerations

Setting up a cytostatic assay should only require about 2-3 hours, depending on the number of compounds tested and the time required to make serial dilutions. Similarly, setting up the Paguio cytocidal assay will require 2-3 hours, depending on the number of compounds assayed. Following the 6 hour incubation, washing of the plates will require 1-2 hours. Incubation of the plates to allow for full growth requires about 72 hours for cytostatic and 48 hours for cytocidal assays.

7.3.8. Acknowledgements

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Aggazzotti, G.; Fantuzzi, G.; Righi, E.; Predieri, G. Occupational and environmental exposure to perchloroethylene (PCE) in dry cleaners and their family members. *Arch Environ Health* 1994, 49, 487-492.


Bhattacharya, A.; Mishra, L. C.; Sharma, M.; Awasthi, S. K.; Bhasin, V. K. Antimalarial pharmacodynamics of chalcone derivatives in combination with artemisinin against


Brennand, A.; Gualdrón-López, M.; Coppens, I.; Rigden, D. J.; Ginger, M. L.; Michels, P. A. M.


Cooper, R. A.; Lane, K. D.; Deng, B.; Mu, J.; Patel, J. J.; Wellems, T. E.; Su, X.; Ferdig, M. T. Mutations in transmembrane domains 1, 4 and 9 of the Plasmodium falciparum


Damme, K.; Nies, A. T.; Schaeffeler, E.; Schwab, M.


Egan, T. J. Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation. *Journal of Inorganic Biochemistry*. **2008**, *102*, 1288-1299.


Gorka, A. P.; Sherlach, K. S.; de Dios, A. C.; Roepe, P. D. Relative to Quinine and Quinidine, Their 9-Epimers Exhibit Decreased Cytostatic Activity and Altered Heme Binding but


Geneva, Switzerland.


Kennedy, D. C.; McKay, C. S.; Legault, M. C. B.; Danielson, D. C.; Blake, J. A.; Pegoraro, A. F.; Stolow, A.; Mester, Z.; Pezacki, J. P. Cellular Consequences of Copper Complexes

Khalil, I. F.; Alifrangis, M.; Recke, C.; Hoegberg, L. C.; Ronn, A.; Bygbjerg, I. C.; Koch, C.

Development of ELISA-based methods to measure the anti-malarial drug chloroquine in plasma and in pharmaceutical formulations. *Malaria J.* 2011, 10, 249-255.


Knight, S. D.; Adams, N. D.; Burgess, J. L.; Chaudhari, A. M.; Darcy, M. G.; Donatelli, C. A.;


Makanga, M.; Krudsood, S. The clinical efficacy of artemether/lumefantrine (Coartem®).


Parapini, S.; Basilico, N.; Pasini, E.; Egan, T. J.; Olliaro, P.; Taramelli, D.; Monti, D.


Poet, T. S.; Weitz, K. K.; Gies, R. Z.; Edwards, J. A.; Thrall, K. D.; Corley, R. A.; Tanojo, H.; Hui, X.; Maibach, H. I.; Wester, R. C. PBPK modeling of the percutaneous absorption of


Roepe, P. D. To Kill or Not to Kill, That is the Question: Cytocidal Chloroquine Resistance. *Trends Parasitol*. **2014**, *30*, 130-135.


Tawk, L.; Chicanne, G.; Dubremetz, J. F.; Richard, V.; Payrastre, B.; Vial, H. J.; Roy, C.;


Thriemer, K.; Khan, W. A.; Starzengruber, P.; Haque, R.; Vossen, M. G.; Marma, A. S. P.;


Tomlins, A. M.; Ben-Rached, F.; Williams, R. A. M.; Proto, W. R.; Coppens, I.; Ruch, U.;


van Helvoort, A.; Smith, A. J.; Sprong, H.; Fritzsche, I.; Schinkel, A. H.; Borst, P.; van Meer, G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell. 1996, 87, 507-517.


