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CHARACTERIZING THE ROLE OF BRANCHED CHAIN KETO ACID
DEHYDROGENASE IN THE MALARIA PARASITE *PLASMODIUM FALCIPARUM*

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ABSTRACT

The protozoan parasite, *Plasmodium falciparum* causes the most virulent form of malaria in humans and threatens the lives of millions of people every year. Although the global mortality from malaria is decreasing, there is a fear that current antimalarial drugs are becoming ineffective due to parasite resistance. This is causing a global health crisis that is creating an intense pressure for the discovery of new antimalarial targets. Many antimetabolites have been very successful antimalarials in the past, leading to the study of unique aspects of parasite metabolism to identify new therapeutic targets. For example, *P. falciparum* is believed to have repurposed the role of branch chain keto acid dehydrogenase (BCKDH) to substitute for a critical pyruvate dehydrogenase (PDH)-like function based on previous studies of BCKDH in other *Plasmodium* spp. This thesis explores the role of BCKDH during the blood stage development of the malaria parasite *P. falciparum*, using both a pharmacological and a genetic approach. In addition to the construction of the plasmids to generate a *bckdh* knockout, we tested the effect of inhibition of the mitochondrial pyruvate carrier with UK-5099, a known potent inhibitor, using liquid-chromatography mass spectrometry (LC-MS). To supplement LC-MS data, asexual parasite growth was observed under UK-5099 for four days. The results of UK-5099 reveal the immediate flexibility of the parasite's metabolism in the face of metabolite deficient conditions. However, asexual parasites underwent morphological changes and showed signs of death when UK-5099 induced acetyl-CoA deficiency was extended over four days. Therefore, we believe that BCKDH has a PDH-like function in *P. falciparum* and based on the results of the UK-5099 experiments, it is assumed that without the function of BCKDH, the parasite's immediate changes in metabolic flux would not be capable of supporting long-term parasite survival. This provides evidence that BCKDH could be a potential drug target for antimalarial drugs.

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Chapter 1

An Introduction to the study

Plasmodium falciparum is an apicomplexan parasite that causes the most virulent form of malaria in humans and is solely responsible for over 200 million clinical cases of malaria (Loy et al., 2016). The study of *P. falciparum* is becoming more vital as parasites are becoming resistant to all antimalarial drugs, including the first-line treatment artemisinin combination therapy (Dondrop et al., 2009). Therefore, there is a sense of urgency within the malaria community for the discovery of new antimalarial drug targets; and the differences between the human host and *Plasmodium* metabolism provide the strong basis for the study of metabolic pathways for the discovery of new drug targets (Ke et al., 2015)(Oppenheim et al., 2014).

Plasmodium spp. must salvage metabolites from their host in order to survive, which provides the opportunity to target the essential metabolite acquisition processes within the parasite to inhibit vital nutrients from reaching the metabolism (Seheibel and Pflaum., 1970). Metabolic targets are also being explored within various organelles such as the apicoplast, a non-photosynthetic plastid organelle and the mitochondrion, which is the target of the current antimalarial atovaquone. Within the mitochondrion, there are important deviations between human and parasite TCA metabolism. While humans use the majority of glucose for aerobic respiration, parasites use between 80-90% of their glucose for glycolysis. Glycolysis is also parasites main energy source and drives ATP synthesis (Olszewski et al., 2010). Despite their reliance on glycolysis, *Plasmodium* spp. are known to have tricarboxylic acid (TCA) metabolism.

Plasmodium parasites encode for pyruvate dehydrogenase (PDH) however, it is located in the apicoplast, not in the mitochondrion as it is in humans. Also, the acetyl-CoA produced by the PDH complex does not appreciably contribute to the pool of acetyl-CoA in the mitochondria, meaning there is another mitochondrial enzyme responsible for PDH's canonical function (Cobbold et al., 2014). In other apicomplexa parasites, branched chain keto acid dehydrogenase (BCKDH) has the role of mitochondrial PDH and BCKDH knockout parasites were viable but demonstrate significant decreases in growth, virulence, and oocyte development (Oppenheim et al., 2014). This suggests that if BCKDH has the same function in *P. falciparum*—the deadliest malaria parasite—it could have the potential to be an antimalarial drug target.

Therefore, my thesis studied the role of BCKDH in *P. falciparum* parasites by evaluating the function of the enzyme through both a genetic and pharmacologic approach. The genetic approach involved the creation of plasmids to be used to generate *bckdh* knockout parasites, which would allow for the direct study of the enzyme's function. The pharmacologic approach used the potent mitochondrial pyruvate inhibitor, UK-5099, to study the effect of acetyl-CoA depletion on *P. falciparum* parasites. Trophozoite staged parasites were extracted and treated with UK-5099 and then analyzed using liquid chromatography-mass spectrometry (LC-MS). In addition to metabolomics, asexual parasites were treated with UK-5099 over the course of two life cycles to monitor physiological and morphological changes that occurred under the drug.

Further insight into essential metabolic functions for gametocyte-stage parasites was accomplished through metabolomic experiments comparing human serum and Albumax II, two medium supplements used for culturing parasites. The results were then incorporated into the main objective of this thesis, which was to further explore the role of BCKDH in *P. falciparum* metabolism and assess the effect of acetyl-CoA depletion on asexual parasite development.

Chapter 2

Literature Review

Background

Malaria continues to threaten the lives 3.3 billion people in 91 countries worldwide, despite discoveries of ancient antimalarials, global eradication initiatives, and identification of new drug targets (WHO., 2016). Malaria is a vector borne disease caused by parasites in the *Plasmodium* genus, which comprises over 250 species that cause malaria in different vertebrates. However, only 6 species of *Plasmodium* can cause disease in humans (Cowman et al., 2016). While *P. falciparum* is considered the deadliest human malaria parasite worldwide, *P. vivax* causes the majority of malaria cases in Asia and Latin American (Naing et al., 2014). *P. ovale curtisi*, *P. ovale wallikeri*, and *P. malariae* also cause malaria in humans but to a much lesser extent than *P. falciparum* and *P. vivax*. *P. knowlesi* is considered a new emerging parasite in Southeast Asia; although it remains mainly zoonotic and cannot be transmitted human to human (Ahmed and Cox-Singh., 2015). *P. falciparum* is the most prevalent malaria species in sub-Saharan Africa and annually causes 200 million clinical cases and over 300,000 malaria-related deaths, with the highest population of deaths in children under five (Loy et al, 2016).

P. falciparum's deadly pathogenesis can be attributed to its fundamental characteristics such as exponential parasite growth, stimulation of host inflammatory responses, and microvascular obstruction leading to endothelium activation (Cowman et al., 2016). Malaria's characteristic symptom is a periodic fever that corresponds to the life cycle of the parasite (Kwiatkowski and Greenwood, 1989). The severity of symptoms, however, can range substantially leading to two different classifications. Malaria is classified as either uncomplicated

malaria or severe malaria (Bartoloni and Zammarchi., 2012). The clinical manifestations of uncomplicated *P. falciparum* infection “include hypoglycemia, lactic acidosis, hemolytic anemia, hemoglobinuria, and hypoargininemia” (Olszewski et al., 2009). In addition to these paramount symptoms of disease, *P. falciparum* infections have the ability to progress to a more robust infection called severe malaria. Severe malaria occurs in 1-2% of cases and the population at risk varies depending on the level of malaria transmission in a given geographical area. High transmission levels are associated with severe malaria in children 1 to 4 years old; however, in areas with lower transmission it is more common for adults to develop severe malaria (Wassmer et al., 2015)

Immune Response

Severe malaria is characterized by three hallmark symptoms in the human host: cerebral malaria, acidosis/respiratory distress, and severe anemia (Cunnington et al., 2013). The lack of animal models that display severe malaria in the same way humans do has contributed to the difficulty of determining the exact pathogenesis that leads to these severe and deadly symptoms caused by a *P. falciparum* infection (Craig et al., 2012). The absence of a defined pathogenesis has led to many theories about what causes the progression to severe malaria. One theory states that during a malaria infection, red blood cells infected with both sexual and asexual parasites (iRBC) are sequestered in the small vascular of the body, causing a microvascular obstruction that progresses to severe malaria (Cunnington et al., 2013). The vasculature of the body is lined with endothelial cells, which are important for the regulation of blood flow, barrier functions, and coagulation. When iRBCs begin to build up in the vascular endothelium, catastrophic

consequences can arise from disturbances in nitric oxide bioavailability (Cunnington et al., 2013). In addition to the adherence of iRBC to the vascular wall, the release of proinflammatory cytokines, hemoglobin, glycosylphosphatidylinositol, and histones can all lead to the loss of the barrier function, dysfunction of coagulation, and amplification of the inflammatory response (Gillrie et al., 2012). This highlights the breakdown of the endothelium as a potential mechanism for severe malaria and links inflammation and iRBC sequestration, which ultimately leads to ischemia in tissues (Cunnington et al., 2013)

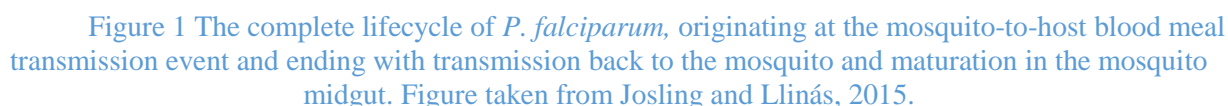
In addition to microvascular obstruction, cerebral malaria is another distinctive characteristic of severe malaria that results from increased permeability of the blood brain barrier. *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*) is the most characterized adhesion protein in *Plasmodium* and is responsible for parasites binding to host endothelial cells. *PfEMP1* is encoded from the family of *var* genes, which have developed a unique way of evading the host immune system by enabling the parasite to periodically change which gene is expressed. This is known as antigenic variation and it is vital for proliferation and transmission of malaria parasites as it hinders the host's ability to generate an immune response (Flick and Chen, 2004). *PfEMP1* in late asexual parasites binds to host receptors in the vascular endothelium of various organs including liver, kidneys, and the blood brain barrier leading to multisystem disorder (Wassmer et al, 2015). When comparing patients with cerebral malaria to patients with uncomplicated malaria, *PfEMP1* binds to the human cerebral microvascular endothelium with increased strength and frequency, which might result in the alternation of the blood brain barrier and eventually cause cerebral malaria (Claessens et al., 2012). Pro-inflammatory cytokines such as tumor necrosis factor (TNF- α), interleukin-6 (IL-6), and IL-10 levels were found to be significantly higher in patients with cerebral malaria compared to

patients with uncomplicated malaria. Although, there was a lot of variation in the results and the exact relationship between cytokines and cerebral malaria cannot be determined (Kwiatkowski and Greenwood., 1990).

In addition to the microvascular theory for severe malaria, another potential cause of severe malaria is the exponential growth increase of malaria parasites within the human host. The rapid increase in the amount of parasites in the blood could cause the body to have an excessive immune response that causes the symptoms of severe malaria (Cunnington et al., 2013). Death from severe malaria is strongly associated with brain swelling, or vasogenic edema, in children as a result of the blood brain barrier disruptions (Seydel et al., 2015). Instead of one specific issue such as high parasitemia and microvascular obstruction, it is possible that severe malaria is caused by a cascade of many events. High parasitemia could increase the occurrence of iRBC sequestration in microvascular which causes endothelium destruction that promotes an excessive inflammatory response. A high inflammatory response is evident by increased swelling and cytokines IL-6, IL-10, TNF- α (Kwiatkowski and Greenwood, 1990). Vascular pathology has been found in patients that died from severe malaria once again solidifying the link between the dysregulation of the endothelium as a result of iRBC binding to the blood vessels, which propagates inflammation as well as the effects of endothelium destruction (Seydel et al., 2015). The lack of a clear pathogenesis of *P. falciparum* is not the only factor that has hindered the control and eradication of malaria.

Life Cycle

One of the major complications in creating a vaccine or discovering a new antimalarial drug is *Plasmodium*'s complex life cycle. During its lifecycle, *Plasmodium falciparum* alternates between the human host and its vector, the female *Anopheles* mosquitoes. During a blood feed, mosquitoes transmit sporozoite-stage parasites into the dermis of the host (Figure 1A) (Cowman et al., 2016). Over the next 1 to 3 hours, parasites make the journey from the site of transmission to the liver. The beginning of the journey to the liver starts with the random process of gliding motility, which allows a proportion of parasites to exit the dermis, travel to dermal blood vessels, and arrive at the bloodstream, where they enter the circulatory system. The less fortunate parasites that lack the ability to do gliding motility are broken down and sent to the lymph nodes where they are cleared by the host immune system. The second half of the journey involves parasites arriving at the liver's sinusoidal barrier and a process called cell traversal (Cowman et al., 2016). Overall, cell traversal is a broad term used to describe sporozoites ability to gain access to a host cell, continue through the cytosol and exit the host cell (Mota et al., 2001). In order to gain access to hepatocytes, sporozoites must traverse the sinusoidal barrier by entering either endothelial cells or Kupffer cells (resident macrophages in the liver) (Tavares et al., 2013). Once parasites have infected hepatocytes, they begin their progression to the next stage of the life cycle (Figure 1B). For about 2-10 days, parasites enter into the liver stage of the life cycle and prepare to release up to 40,000 merozoites, which will exit the liver through hepatic circulation and invade red blood cells to establish an erythrocyte infection (Strum et al., 2006). The process for invading erythrocytes only takes 2 minutes but it involves multiple steps including pre-invasion, internalization, and echinocytosis (Weiss et al., 2015).



Gametocyte development is very distinct from asexual development. Gametocytes do not undergo any replication and are arrested from the moment they invade a red blood cell. In *P. falciparum*, gametocytes undergo five morphological stages of development (Figure 2) over the course of 10-12 days (Josling and Llinás., 2015). The development of gametocytes is marked by distinct morphological changes. As the gametocyte develops, it progresses from a trophozoite-like shape to a crescent shape that molds the RBC as it grows. Gametocytes can also be easily distinguished from asexual parasites because of the presence of osmiophilic bodies within the parasite (Figure 2). Stages I-IV occur in the bone marrow to avoid clearance from the spleen, but stage V can be found in the bloodstream as it needs to be available to a mosquito to ensure transmission (Guttery et al., 2015).

Stage V, gametocytes are able to survive in the human body for over a month while waiting a transmission event (Bousema et al., 2010). Once gametocytes are taken up into the midgut of the mosquito, they mature into gametes. In the course of 20 minutes, a male gametocyte must undergo three rounds of DNA replication followed by the development of 8 flagella to become a mature male gamete (Janse et al., 1987) that is able to fuse to a female gamete to produce a diploid zygote (Sinden.,1983). Then the zygote matures into an ookinete and undergoes subsequent meiosis. In order to sustain an infection in the mosquito ookinetes must invade the epithelial cell wall of the midgut to mature into an oocyte. These oocytes will develop into infectious sporozoites that rupture and migrate to the salivary glands of the

mosquitoes where they wait for the next human blood meal and restart the cycle within the human dermis (Figure 1D) (Aly et al., 2009).

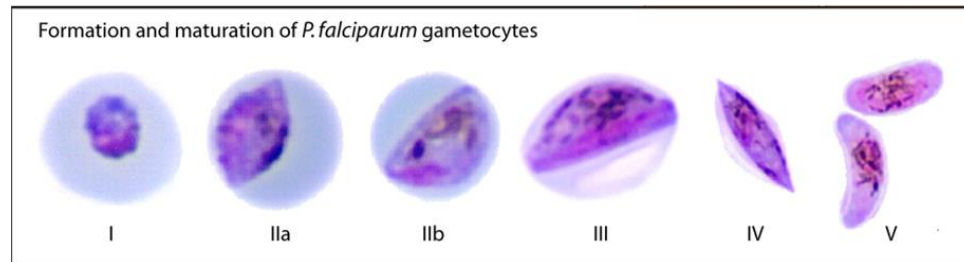


Figure 2 The stages of *P. falciparum* gametocytogenesis. Gametocytes will remain within the bone marrow for stages I-IV and then migrate to the periphery during stage V. Osmophilic bodies start to become noticeable in late stage II. Figure from Bousema and Drakeley., 2011.

Overall, the decision to switch from an asexual stage parasite to sexual stage parasite is a random process in *Plasmodium*. However, the developmental switch has been linked to several environmental factors (Sinha et al., 2014). In the laboratory, gametocyte induction occurs when the parasite is “stressed” under conditions such as high parasitemia or exposure to antimalarial drugs. Although these environmental conditions can cause gametocytogenesis in nature, the correlation is not always 100% (Seidlein et al., 2001). There is evidence that environmental factors are sensed by parasites via cell-cell communication through extracellular vesicles released from *P. falciparum*-infected RBCs. Exosomes can be used as a vehicle to carry nucleic acids from parasite to parasite to induce different developmental changes (Belting and Wittrup., 2008). This leads to increased parasite survival in the host during nonstressful and stressful environmental changes, and maximizes transmission events to the mosquito vector if the host environment becomes too hostile (Regev-Rudzski et al., 2013). Epigenetics also play an important role in *P. falciparum* sexual differentiation. The master regulator of gametocytogenesis is the Apicomplexan AP2 (ApiAP2) domain containing transcription factor AP2-G (Kafsack et al., 2013). AP2-G is part of the apiAP2 transcription factor family, which

comprises of 27 members, some of which have been identified to be important for stage-specific development of *Plasmodium*. In addition to AP2-G, AP2-G2 is linked to parasite development into stages necessary for transmission. AP2-G is unique in the fact that it determines developmental fate, while AP2G2's function is after sexual commitment has taken place (Shinha et al., 2014). The continuation of research on gametocyte epigenetics, life cycle, and differentiation is extremely important for malaria research because of the vitality of gametocytogenesis for transmission to the mosquito vector.

Metabolism

For many years, the parasite's metabolism remained largely unknown due to difficulty of culturing malaria parasites in the laboratory (Trager and Jensen., 1976). Since the development of new culturing methods such as Albumax II, as a human serum supplement, the inner workings of *Plasmodium* spp. metabolism are starting to be uncovered. As an intracellular parasite, *P. falciparum* scavenges its host's carbon sources and essential nutrients such as amino acids, glucose, and glutamine to undergo essential biochemical pathways for survival (Seheibel and Pflaum., 1970). The breakdown of glucose by either anaerobic or aerobic pathways is vital for most organisms and *P. falciparum* is no exception. The first indications of glucose consumption in asexual parasites came from studies that found that RBCs infected with asexual parasites consumed 50 to 100-fold the amount of glucose as uninfected red blood cells (Roth E Jr, 1990). The amount of glucose consumed in iRBC reaches 100-fold during the most metabolically demanding stages, trophozoite and schizont stages; however, glucose is mostly broken down through the anaerobic fermentation pathway to lactate, which is excreted from the cell (Scheibel

and Pflaum, 1970). This initial finding led researchers to infer for many years that parasites did not have TCA metabolism, but that was eventually proven false (Olszewski et al., 2010) (Ke et al., 2015). The specifics of *Plasmodium* TCA metabolism will be discussed later and for now it is important to note the differences between other metabolic processes.

Although glycolysis is the main fate glucose in parasites, intermediates can be shuttled to other processes such as the pentose phosphate pathway (PPP) and the synthesis of nucleic acids. *P. falciparum* are known to perform the PPP and encode all enzymes necessary except transaldolase, an enzyme in the canonical PPP (Bozdech et al., 2003). The pentose phosphate pathway is responsible for generating NADPH and the five carbon sugars that are needed to synthesize nucleic acids for DNA synthesis. The pentose phosphate pathway is active at different levels throughout the parasites life cycle peaking at the trophozoite stage, which is increased 78-fold during this stage (Atamna, 1994). This corresponds to the overall heightened metabolic demand of trophozoite parasites (Cobbold et al., 2013)

While parasites have the ability to perform the PPP like humans, it is believed that parasites do not store, conserve, or generate glucose (gluconeogenesis) like in the human host. This conclusion is due to the absence of genes that encode essential enzymes. However, parasites have conserved the genes that encode for phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC). Traditionally, PEPCK is responsible for converting oxaloacetate and ATP to phosphoenolpyruvate (PEP) and carbon dioxide for gluconeogenesis and PEPC catalyzes the reverse reaction (Hayward, 2000). PEPCK and PEPC have opposite gene expression profiles in *Plasmodium*, leading researches to believe these enzymes have their canonical functions in *Plasmodium*, despite original assumptions that parasites do not perform gluconeogenesis (Bozdech et al 2003). The gene that encodes PEPCK is unregulated during the

developmental switch for gametocytogenesis as well as the ensuing gametocyte stages, implying that if parasites did perform gluconeogenesis, it could be important for these stages (Hayward, 2000). Additional research must be performed to determine what the true function of PEPC and PEPC is and whether parasites can perform gluconeogenesis.

Another metabolic difference between humans and *Plasmodium* is the role of fatty acid synthesis. While the true function of fatty acid II biosynthesis in *Plasmodium falciparum* has not been determined, it is known that it is not similar to fatty acid synthesis in humans (van Schaijk et al., 2014). Fatty acid synthesis in *P. falciparum* occurs in the apicoplast (Waller et al., 2003) not the cytoplasm and it does not have the function of storing long-term energy when there is abundant glucose, as in humans (Voet et al., 2012). In *P. falciparum*, fatty acids can either be salvaged from the host or synthesized through fatty acid II biosynthesis which involves a different mechanism than humans (van Schaijk et al., 2014). Overall, the differences between human and *P. falciparum* metabolism has led scientists to turn their focus to metabolic enzymes and compounds for possible drug targets. In one study, flux balance analysis was used to evaluate metabolic genes that could encode potential antimalarial drug targets. The results found 16 metabolic protein genes that had no significant sequence identity to any human genes. In addition, 5 of those 16 genes are not present at all in humans indicating promise as drug targets within the underexplored metabolism (Plata et al., 2010).

Beyond the metabolic differences between the human and parasite, there are also significant differences in metabolism between parasite life stages. Compared to gametocytes, asexual parasites are much less reliant on the TCA cycle (Bozdech et al., 2003). This is apparent since six out of the eight mitochondrial enzymes can be deleted without asexual growth defects (Ke et al., 2015). In fact, phenotypes in knockout parasites did not manifest until the gametocyte

or mosquito stage, highlighting gametocytes' increased dependence on the mitochondria TCA cycle. Asexual parasites' ability to withstand TCA enzyme knockouts sheds light on the ability of asexual parasites to adjust their metabolic flux to account for adverse environments. For example, when enzymes important for the generation of glucose-derived intermediates were knocked out of the parasites genome, glutamine fed into the TCA at an increased rate (Ke et al., 2015). These changes in carbon flux ensure that the latter part of the TCA cycle is always completed despite decreases in glucose or glutamine. On the other hand, gametocytes were not able to manipulate their metabolism to the same extent as asexual parasites and TCA enzyme knockout gametocytes were unable to complete maturation.

In agreement with the discovery that gametocytes rely more on TCA metabolism, gametocytes have been found to have increased expression levels of α -ketoacid dehydrogenase complexes (KADH), which are important class of metabolic enzymes for the TCA cycle (McMillan et al., 2013). While KADH enzymes expression is elevated in gametocytes, the enzymes are still present in all stages of *P. falciparum* parasites (McMillan et al, 2013). All KADH enzymes are multienzyme complexes with three subunits, E1 is decarboxylase, E2 is dihydrolipoamide transacylase, and E3 is dihydrolipoamide dehydrogenase (LipDH). The following are all important KADHs: Pyruvate dehydrogenase (PDH) (Figure 3), branched chain keto acid dehydrogenase (BCKDH), and ketoglutarate dehydrogenase (KGDH). The *P. falciparum* genome contains all of the genes encoding PDH, BCKDH, and KGDH, which sparked researcher's attention to the mitochondrial metabolic processes (McMillan et al., 2005).

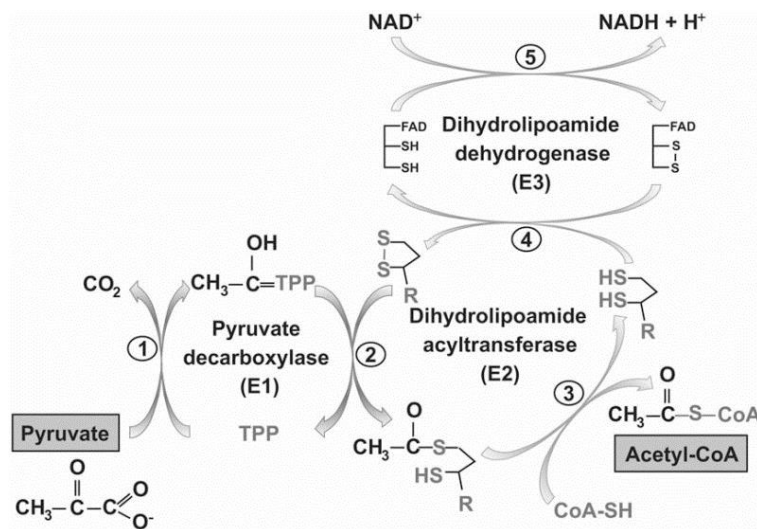


Figure 3 This is a schematic of pyruvate dehydrogenase's catalytic mechanism for converting pyruvate into acetyl-CoA. While all KADH enzymes have unique mechanisms, the characteristic three-subunit structure with E1, E2, and E3 can be clearly seen in this figure. Figure from Storm and Müller 2012.

In most organisms, pyruvate, which is derived from glycolysis, is converted into acetyl-CoA via the PDH complex located in the mitochondrial matrix and subsequently fed into the mitochondrial TCA cycle. PDH uses the cofactors thiamine, NAD^+ , and lipoic acid to combine pyruvate and co-enzyme A to form acetyl-CoA. Traditionally, the outcome of the TCA cycle is NADH, which fuels the generation of ATP through the electron transport chain. However, *P. falciparum*'s PDH complex is not located in the mitochondria, but rather is found in the apicoplast, a plastid-like organelle responsible for fatty-acid synthesis. Also, *Plasmodium* spp. generate all ATP from glycolysis without the electron transport chain (McMillan et al., 2005). Regardless of the difference in parasites' ATP generation, NADPH is still important for the transfer of electrons to the mitochondrial electron transport chain; however, the electron transport chain has been repurposed to fuel pyrimidine biosynthesis rather than ATP production (Painter et al., 2007). Parasites are not able to salvage pyrimidines from red blood cells therefore, the ability to do pyrimidine biosynthesis is vital for survival and replication, increasing the

importance of the TCA cycle (Painter et al., 2007). The unique location of PDH in *P. falciparum*, has led to the in-depth study of the enzyme.

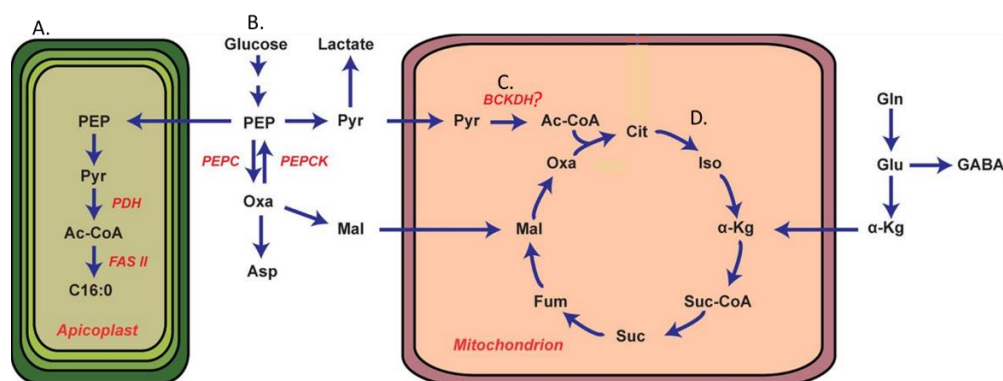


Figure 4 An overview of the *P. falciparum* metabolism highlighting the two acetyl-CoA pools, one in the apicoplast and one in the mitochondria. Figure from Cobbold et al., 2013.

PDH knockout parasites were generated to assess the contribution of acetyl-CoA formed by the PDH complex (Figure 4A) to the pool of acetyl-CoA in the mitochondria (Cobbold et al., 2013). The E1 subunit of the PDH complex is critical for the function of the complex and controls the thiamine pyrophosphate dependent decarboxylation of pyruvate, and was therefore knockout of *P. falciparum* genome to render the enzyme nonfunctional. [¹³C] glucose labeling was used to track the flow of carbons through glycolysis and the TCA cycle to assess the effects of a non-functional PDH complex. The study concluded that parasites that lacked PDH function showed no significant difference in mitochondrial acetyl-CoA, implying that the PDH complex does not applicably contribute to mitochondrial acetyl-CoA (Cobbold et al., 2013).

Due to the lack of altered metabolic flux in knockout PDH complex blood stage parasites, the effect of the knockout was then tested in gametocytes. Gametocytes with the disruption of PDH did not have a change in gametocytogenesis, exflagellation, or oocyst development. In the mosquito midgut, however, PDH deficient oocysts were unable to progress and produce sporozoites (Cobbold et al., 2013). These conclusions implied that another enzyme must be

responsible for the PDH-function in the mitochondrion. To assess whether another KADH enzyme was responsible for this role, knockout parasites and wild type parasites were treated with oxythiamine, which is a broad spectrum inhibitor of thiamine pyrophosphate-dependent reactions. Surprisingly, both the PDH knockout parasites and wild type parasites experienced a 70-80% inhibition of acetyl-CoA, indicating that another thiamine pyrophosphate-dependent reaction was responsible for converting pyruvate to acetyl-CoA. *P. falciparum* parasites have three other pyrophosphate-dependent enzymes: transketolase, KGDH, and BCKDH.

Transketolase and KGDH both have defined roles in the pentose phosphate pathway and TCA cycle respectively in *P. falciparum*, therefore attention was turned to BCKDH. Also, BCKDH did not have its normal function in *P. falciparum* when it was tested. In conclusion, this study proposed for the first time that branched chain keto acid dehydrogenase (BCKDH), a thiamine pyrophosphate-dependent enzyme located in the mitochondria, may have the role of PDH in *P. falciparum* (Figure 4C) (Cobbold et al, 2013).

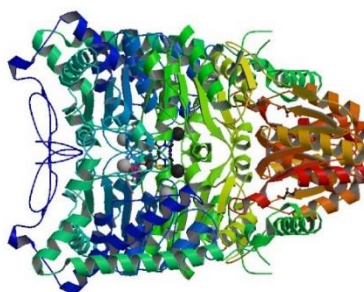


Figure 5 The ribbon diagram for the human mitochondrial branched chain keto acid dehydrogenase (RCSB Protein Data Bank)

BCKDH (Figure 5) is a multi-subunit complex that is normally part of the branch chain amino acid catabolic pathway in the mitochondria. It breaks down valine, leucine, and isoleucine via oxidative decarboxylation resulting in the generation of acetyl-CoA along with other acyl-

CoAs and acetoacetate in humans (Shimonmura et al., 2015). Although *P. falciparum* parasites do not use BCKDH for its normal catabolic function, the enzyme is actively expressed during the erythrocytic stage for development (Cobbold et al., 2013). BCKDH and PDH are also functionally similar, further indicating that BCKDH has PDH-like function in parasites (Oppenheim et al., 2014). The original experiments by Cobbold that implied BCKDH is the missing link, failed to experimentally isolate BCKDH as the enzyme that converts pyruvate to acetyl-CoA. Cobbold, used oxythiamine, which inhibits all thiamine pyrophosphate-dependent reactions, to evaluate what happened to the acetyl-CoA pools. Although KGDH and transketolase have defined roles in the parasite, the decrease in acetyl-CoA production in the presence of oxythiamine cannot be assumed to be solely attributed to BCKDH.

In a subsequent study, Oppenheim furthered explored the role of BCKDH by conducting experiments that directly evaluated the enzyme's function (Oppenheim et al., 2014). To study the role of BCDKH in apicomplexan parasites, *Toxoplasma gondii* and *Plasmodium berghei* were used to assess the phenotype of a BCKDH knockout parasite. *T. gondii* is a very common parasite in developed countries, but it is asymptomatic in immune competent individuals (CDC). *P. berghei* causes rodent malaria and it is commonly used to study malaria in laboratory experiments. In order to functional disrupt BCKDH, the E1 α subunit of the protein was knocked out by double homologous recombination. In humans, mutations of the E1 α subunit are sufficient to render catalytic activity of the protein ineffective indicating the same for *P. falciparum* (Oppenheim et al., 2014). *P. berghei* is a *Plasmodium* parasite therefore, the results of the BCKDH deletion are more pertinent for this thesis.

P. berghei knockout parasites exhibited such a significant fitness loss that they were unable to successfully infect immunocompetent mice. Therefore, the parasites had to be grown in

immunodeficient mice yet they still grew at a slow rate, which resulted in much lower parasitemia levels compared to wild type. Upon further investigation, it was noticed that BCKDH knockout *P.berghei* parasites lacked the ability to develop in normocyte red blood cells and could only maintain an infection in reticulocytes, which are immature red blood cells. Compared to normocytes, reticulocytes are known to have an increased amount of acetate, which can be used by parasites to create acetyl-CoA in the absence of BCKDH-derived acetyl-CoA (Oppenheim et al., 2014). Therefore, the excess acetate would have offered a compensatory mechanism for knockout parasites. Once parasites were supplemented with acetate they were able to partially grow in normocytes. It was confirmed that in *P. berghei*, BCKDH does have a PDH-like function and knockout parasites experienced a decrease in TCA glucose-derived metabolites. Lastly, BCKDH is critical for parasites to develop into mature gametocytes and is essential for oocysts to mature and undergo sporogony (Oppenheim et al., 2014). These experiments revealed that BCKDH not only has a PDH-like function in *T. gondii* and *P. berghei*, but also is strongly intertwined with many important processes in parasites including virulence, infectivity, and fitness. This highlights the potential for BCKDH to be a viable drug target for malaria. *P. berghei* and *T. gondii* are not identical in *P. falciparum*, however, and the experiments performed by Oppenheim must be replicated in *P. falciparum* to confirm the function of BCKDH. The genes that encode BCKDH are conserved across the species indicating that the function in *P. falciparum* may be similar to the function in *P. berghei* (Figure 6).

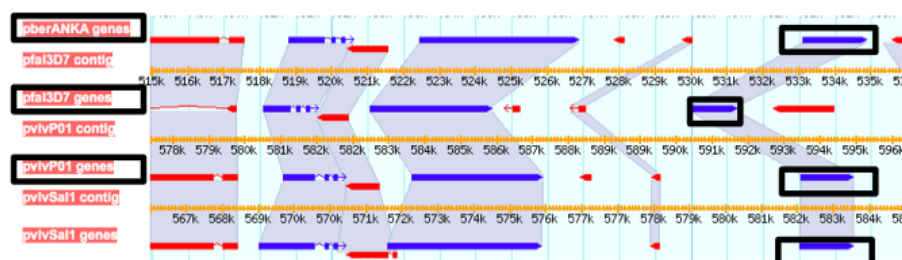


Figure 6 The branched chain keto acid dehydrogenase genomic sequence is strongly conserved between *Plasmodium* spp. The boxed species are *P. falciparum*, *P. berghei*, and *P. vivax* (another common human malaria) (PlasmoDB).

Pharmacology

In 1996, the task of sequencing the whole *P. falciparum* genome was undertaken. During the sequencing process difficulties stemming from *P. falciparum*'s unusually high adenine and thymine (A/T) composition arose and those complications have continued to persist through all experimentation with the parasite. The A/T composition of the genome is 80.6% and rises to 90% in introns and intergenic regions (Gardner et al., 2002), for reference the bacteria *Salmonella* has a genome that is 47.8% A/Ts (Papnikolaou et al., 2009). The high A/T content has added extra complexity to cloning with *P. falciparum*, because primers that are designed to target intergenic regions can lead to nonspecific binding that produces unwanted PCR products. In addition to these limitations, transfecting with *P. falciparum* remains highly inefficient and also involves the use of selection drugs which can have unknown effects on the parasites and should be avoided when working with metabolism (de Koning-Ward et al., 2015). In order to circumvent the complications associated with cloning and creating knockout parasites, pharmacologic inventions that are evaluated using LC-MS can be used (Figure 7) (Cobbold et al, 2013) (Allman et al., 2016). Due to previous literature, a deletion of BCKDH in *P. falciparum* is expected to inhibit the conversion of pyruvate to acetyl-CoA in the mitochondria (Oppenheim et

al., 2014). However, pyruvate begins as a product of glycolysis in the cytosol and must be transported into the mitochondria through the mitochondrial pyruvate carrier (MPC) (Bricker et al., 2012) (Halestrap, 1997). The mitochondrial pyruvate carrier complex is embedded in the inner mitochondria matrix and it is the structure that regulates pyruvate transport (Bricker et al., 2012).

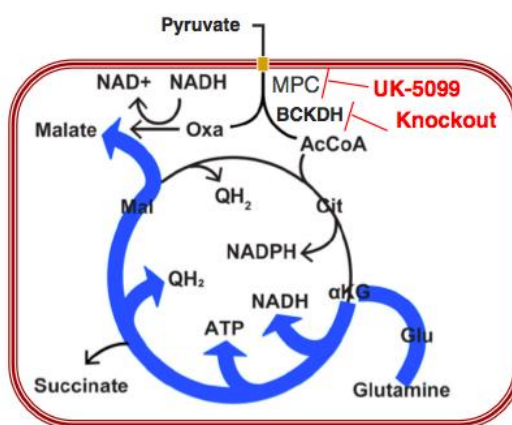


Figure 7 The proposed TCA cycle in *P. falciparum*. The portion of the TCA cycle used most heavily by asexual stage parasites is highlighted in blue. The manipulations of the metabolic pathway that were used to determine the role of BCKDH are highlighted in red. Figure adapted from Ke et al., 2015

Prior to 2012, the existence of the MPC was known from previous experiments; however, the exact molecular makeup of the carrier was not known until 2012 (Herzig et al., 2012). In yeast, the MPC is composed of three subunits MPC1, MPC2, and MPC3. In humans, the MPC is thought to only be comprised of two subunits MPC1 and MPC2, each having three transmembrane alpha helices (figure 8B) (Herzig et al., 2012). Although the gene that encodes for MPC is present in the *P. falciparum* genome, the precise molecular structure of the carrier in *P. falciparum* is unknown (PlasmoBd). In the 1970s, many of the experiments evaluating pyruvate transport into the mitochondria used inhibitors such, α -cyano-4-hydroxyconnamate and its cinnamic acid derivative compounds (CADs) (Halestrap, 1977). In addition to pyruvate carriers, CADs can also target carrier-mediated lactate transport across the erythrocyte

membrane by inhibiting the lactate: H^+ symporter (Kanaani and Ginsburg, 1992). The drug UK-5099 is one of the most potent CADs in *P. falciparum* that targets the MPC (Figure 8A (Halestrap, 1974). UK-5099 inhibition prohibits transport of pyruvate from the cytosol to the mitochondria, which mimics the expected acetyl-CoA disruption in a BCKDH knockout parasite (Figure 8B). UK-5099 arrests parasite growth and results in inhibition of glucose and glycine transport. To a lesser extent lactate transport is also inhibited because of broad CAD activity (Kanaani and Ginsburg, 1992).

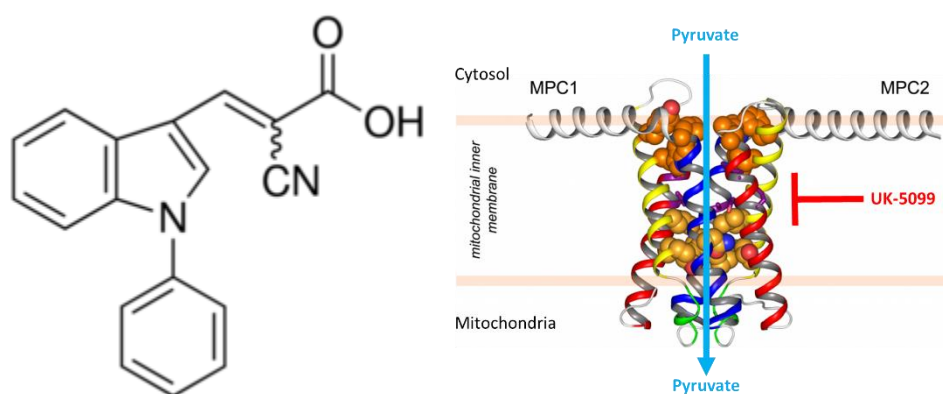


Figure 8 A. The structure of the drug UK-5099. B. The structure and function of the mitochondrial pyruvate carrier in mammals with the inhibition of UK-5099 depicted. Figure from <http://www.mrc-mbu.cam.ac.uk>.

Although, humans and parasites share common metabolic pathways there are enough differences that a potential antimalarial drug targeted to the TCA cycle would have selective toxicity (Ke et al., 2015). The TCA cycle has the ability to be an extremely successful drug target because it is not only vital for gametocytogenesis and transmission to mosquitoes but also could be essential for asexual development if it is targeted at the right location. In addition to traditional knockout experiments, the metabolism can be studied using pharmacology and metabolomics.

Metabolomics

Traditional biochemistry experiments have many limitations that prevent the discovery of what is truly happening within an organism (Li et al., 2008). Metabolomics, the field of study that evaluates changes in molecules and metabolites, has filled this gap and given researchers the ability to glance into the inner workings of an organism (Greef et al., 2013). The start of modern metabolomics took off in the early 20th century using mass spectrometry to measure molecules in biochemical pathways. Early studies in metabolomics shed light on many diseases from alcoholism to schizophrenia and the progression of the field continued with the coupling of gas chromatography (Greef et al., 2013). Since then, metabolomics has been used to study many different organisms (humans, parasites, etc), tissue cultures, pathogens, and clinical patients (Olszewski and Llinás., 2013). It can also provide many different purposes such as; “biomarker identification, diagnosis, disease classification, basic biochemical analysis of metabolic flux and regulatory mechanisms, and understanding the mechanism of drug action and toxicity” (Olszewski and Llinás., 2013). Metabolomics serves as a great technique to study host/parasite mechanisms and has been done in various parasite species such as *Leishmania* and *Toxoplasma* (Kafsack and Llinás., 2010).

In the 1980s, metabolomics was first used to study parasite metabolism using ¹³C-NMR (Chapman et al., 1985). The first metabolomics experiment on the complete intraerythrocytic developmental stage in *Plasmodium* spp. used NMR metabolomics to assess 89 known metabolites over the 48-hour blood life cycle. This study shed light on arginase activity and the effects on arginine depletion in the host during malaria (Olszewski et al., 2009). Metabolomics has also been used to identify biomarkers in *Plasmodium falciparum* that can be used to track the progression of disease, with the hope of creating a more accurate diagnostic technique (Li et al.,

2008). Since then, liquid chromatography has been used for many cutting-edge experiments that have unveiled many of the mysteries of metabolism (Ke et al., 2015). Reverse genetics can be combined with metabolomics to quickly characterize the effects of an enzyme by creating knock out parasites. This process lends itself well to *P. falciparum* because it has a haploid genome (Ke et al., 2015). Even more recently, the Llinás lab coupled pharmacology with metabolomics to study the mode of action of 189 drugs using ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS). The combination of these techniques is groundbreaking and has untold impacts not only with using pharmacologic tools to study the parasite but also increasing the understanding of how antimalarial medications work. This could also advance the understanding behind the mechanisms that lead to drug resistance by measuring the metabolic flux in resistant parasites and comparing levels to susceptible parasites to identify a shift in metabolites (Allman et al., 2016). Continuing to use metabolomics to study *Plasmodium*, will advance the field of malaria and generate new insights on host-parasite interaction, parasite metabolism, the mode of action of drugs, and antimalarial resistance within parasites.

Serum vs Albumax

In order to study erythrocytic stage parasites the perfect culturing conditions had to be established that would allow for continuous cultivation at realistic parasitemia levels. In 1976, the first media that allowed for asexual parasites to be cultured consisted of HEPES-buffered RPMI 1640, supplemented with human serum, erythrocytes, and sodium bicarbonate (Trager and Jensen., 1976). However, it was not long before issues began to arise. Culturing in human serum caused many difficulties with experimentation such as it was expensive, highly variable, and had

the possibility of having contaminating inhibitory immune factors and antimalarial drugs (Schuster., 2002). Since then, Albumax II, a lipid-rich bovine serum albumin has been used as a human serum alternative for cell culture (Thermo Fisher). Albumax II is produced by Thermo Fisher Scientific and it is added to RPMI 1640 for parasite cultivation (Cranmer et al., 1997). Albumax has many advantages over using serum, for example it provides more consistency and eliminates the need for additional serum tests before experiments, saving time and money (Cranmer et al., 1997). A major component of human serum is high amounts of lipids and fatty acids for the parasites to scavenge. Although parasites can perform *de novo* fatty acid synthesis II, fatty acids and lipids must be present in the media to allow parasites to undergo continuous culture (Schaijk et al., 2014). This is the reason why a lipid-rich albumin had to be used as a supplement for human serum. Albumax II is sufficient to allow continuously culture however, gametocytes generated in Albumax II cannot infect mosquitoes. This opens up the opportunity for additional study to determine what the difference between Albumax II and serum metabolites are. The metabolic difference between the two could shed light on a possible antimalarial drug that blocks transmission to mosquitos.

Conclusion

The *P. falciparum* metabolism is becoming clearer and opening many doors to new antimalarial targets along the way (Gardner et al., 2002). There is a major call for new antimalarials as resistance in some countries is on the rise (Cibulskis et al., 2012). Even the first line drug, artemisinin, is now confronted with resistant parasites (Dondorp et al., 2009). The realization that PDH in *P. falciparum* functions in the apicoplast and another enzyme is

responsible for converting pyruvate into acetyl-CoA in the mitochondria, introduces a new potential drug target (McMillan et al., 2005). The mystery enzyme was identified as BCKDH in *P. berghei* and *bckdh* knockout parasites were associated with decreased virulence, growth, and transmission in both the asexual and sexual stage (Oppenheim et al., 2014). While *P. berghei* and *P. falciparum* are in the same genus their genomes are only 86% similar, which means that the knockout experiments performed by Oppenheim, must be repeated in *P. falciparum* to confirm BCKDH's function (NCBI BLAST). However, *P. falciparum*'s high density of A/Ts in the genome can make cloning difficult; therefore, the drug UK-5099 presents a pharmacologic method to assessing the proposed effects of a BCKDH knockout. The combination of pharmacology and metabolomics serves as a technique to visualize what is occurring within the parasite during drug treatment (Kafsack and Llinás., 2010). Also, an antimalarial drug targeting BCKDH has the potential to provide selective toxicity because BCKDH-E1 α in *P. falciparum* is only 41% identical to the human BCKDH-E1 α (Figure S1). I hypothesize that BCKDH has a PDH-like function in *P. falciparum* and *bckdh* knockout parasites will experience decreased virulence, growth, and transmission, presenting BCKDH and its surrounding pathways as a potential antimalarial target. This thesis will evaluate the role of BCKDH through both a genetic and pharmacologic approach.

Chapter 3

Methods and Materials

Overall for All Experiments

Database searches and resources

The gene PF3D7_1212600 was identified in the Plasmodium genomic database PlasmoDB (<http://plasmodb.org/plasmo/>). The gene maps to chromosome 13 in *P. falciparum* 3D7 parasites. The sequence of this gene was taken from PlasmoDB and primers were designed using that DNA sequence. Genomic and protein blasts to compare the *P. falciparum* BCKDH α -subunit sequence to other *Plasmodium* spp. as well as the human sequence was done on NCBI's Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The guide RNAs for CRISPR were made using the website Blencling (<https://blencling.com/academic>). DNA sequencing was sent to GeneWiz, South Plainfield NJ.

Materials

RPMI 1640 used for parasite cultures was purchased from Invitrogen (CAT: 22400105), supplemented with hypoxanthine, gentamycin, HEPES, and 0.25% Albumax II. Designed oligonucleotide primers were ordered from Integrated DNA Technologies, Coralville, Iowa. Restriction enzymes were ordered from New England Biolabs Incorporated. The serum was from anonymous O+ donors (Interstate Blood Bank, Memphis, TN). All blood used in these experiments was collected from anonymous O+ male donors (Biological Specialty Incorporated)

using CDPA anticoagulant. Blood was washed free from CPDA and non-RBC contaminating cell types using Complete RPMI media. The Drug UK-5099 was ordered from Sigma-Aldrich.

Complete *bckdh* Knockout plasmid

Generation of pKP1 and pCas9::sg-bckdh

The *Pfbckdh-e1α* genomic sequence was used to design primers that amplified the 3' and 5' UTR regions of the *bckdh-e1α* gene. The primers 5' UTR F and 5' UTR GC were used to amplify a 992 nucleotide homology region at the 5' end of *bckdh-e1α* gene, starting upstream of the ATG. Once amplified the 5' UTR fragment was cloned between *AatII* and *NcoI* restriction sites in the pHHT-FCU vector, upstream of the *hdhfr* (human dihydrofolate reductase) gene which confers resistance to the drug WR99210. The 3' UTR was amplified using Nested PCR. The inner primers used to amplify the 987 nucleotide homology region at the 3' end of *bckdh-e1α* were 3'UTR F and 3'UTR GC and the outer primers were 3'UTR F2 and 3'UTR GC2. In order to clone the 3' UTR into the pHHT-FCU vector, the FCU region had to be removed with the restriction enzymes *NotI* and *SacII*. The modified vector was denoted as pHHT-FCU(-). The 3' UTR was cloned into the pHHT-FCU(-) vector between the *SpeI* and *NotI* restriction enzymes. Figure 9 shows the primers used to generate the final pKP1 plasmid. The final plasmid, pKP1 was verified with restriction enzyme diagnostics (*NotI* and *MfeI*) and DNA sequencing (Figure 10B).

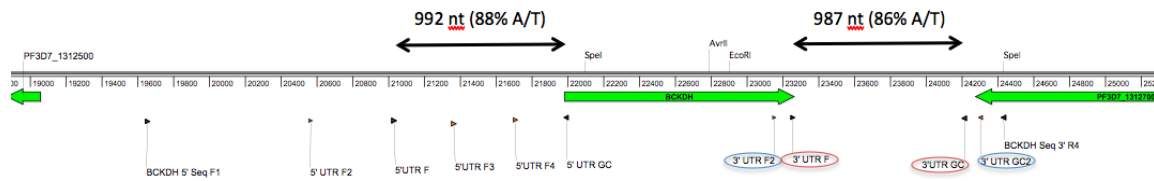


Figure 9 The primers used to generate the final plasmid pKP1. The outside primers for Nested PCR are circled in blue and the inside primers are circled in red.

To increase the probability of a double-strand break the CRISPR-Cas9 system was used. To design the Cas9 plasmid specific for pKP1, two unique guide RNAs (sgRNA) were designed (Blenching, <https://benchling.com/academic>) to target the middle sequence of the *bckdh* gene. The sgRNAs were about 20 nucleotides in length and the sequence was followed with 5' NGG protospacer-adjacent motif (PAM). The oligonucleotides were phosphorylated using PNK and annealed together (Bio Rad Thermocycler) and then subsequently ligated into the plasmid pGEM-U6-B, shuttling vector, that was previously restriction enzyme digested with *Bbs*1. Following sequence validation, the sgRNAs cassette was removed from the shuttling vector and inserted into the final plasmid, pUF1_Cas9 at the restriction site *Not*I. The final Cas9 plasmid was verified via sequencing and diagnostic restriction enzyme digestion (Figure S3).

Transfections with pKP1 and pCas9::sg-bckdh

The pKP1 plasmid and final Cas9::sg-*bckdh* plasmids were electroporated into red blood cells. *P. falciparum* parasites were cultured in RPMI 1640 media and incubated at 37°C and 5% CO₂. Parasites were cultured to about 8% trophozoite parasites in a 50mL flask at 4% hematocrit. Both plasmids were maxi-prepped using a Qiagen maxi-prep kit. The maxi DNA was run on a gel to verify supercoiled DNA and was determined to have concentrations between OD_{260/20} (1.8-2.0) and OD_{260/230} (>2.0). Maxi DNA was aliquoted to 50ng per tube and ethanol

precipitated. Two transfections took place, one with a total of 100ng of pKP1 and one with a total of 100ng of both pCas9::*sg-bckdh* and pKP1. 150µl of RBCs were washed three times with 5-10 volumes of cytomix and added to 150µl of cytomix after the last wash and then electroporated with DNA at 0.31Kv, 960µFD. After electroporation, 100µL of ~8% trophozoite containing blood was added to the electroporated blood in a flask with 10mL of media and returned to the incubator. Two days after electroporation, selection drug media was added to the parasite cultures and continued to be used until parasites were observed. Cultures with only pKP1 were treated with media containing 25µM of WR99210. Parasites transfected with pCas9::*sg-bckdh* and pKP1 were treated with 25µM of WR99210 and 15µM of the drug DSMI. Parasites successfully transfected with the pKP1 and pCas9::*sg-bckdh* have yet to be obtained but the transfection process has been repeated many times with slight variations in procedure. Parasite transfection by invasion of DNA-loaded erythrocytes (adapted from K. Deitsh 2001 NAR) was used most frequently. The other method was a direct electroporation to rings at ~5% parasitemia with 50ng of DNA. 300µl of iRBCs were washed three times with 5-10 volumes of cytomix and then cytomix was added so RBC were at 50% of volume. 250µl of iRBCs was added to 50ng of DNA in 100µl of cytomix and electroporated at 0.31Kv, 960µFC. After electroporation iRBCs were added to a flask with 300µl of fresh RBCs and 10mL of media. Parasites were treated with the same selection drugs 2 days after transfection. DNA concentrations were increased from 50ng to 100ng per electroporation although, no change in transfection was noticed. Selection drug concentrations were also decreased slightly and then increased back to standard concentration after no change was observed. The last change required adding selection drug the day after electroporation with the RBC DNA loading method and then only keeping selection drug on for 6 days.

Acetate Testing

In the absence of the gene *bckdh*, it is assumed that to remain viable, parasites would have to be treated with acetate to generate greater amounts of acetyl-CoA necessary for survival. Before acetate is added to experimental parasites it was necessary to determine whether acetate had an effect on the parasites. The parasites were split into a control group and a group that received acetate supplementation. Parasites were synchronized to rings using 5% sorbital and reduced to 1% parasitemia of rings. The parasitemia of both groups was taken over the course of three life cycles which is equivalent to seven days. Parasites were reduced as needed to prevent lethal parasitemia (>10%=lethal).

Albumax II versus Human Serum

Preparation of Albumax II

The metabolites of Albumax and serum (O+ blood) could not be compared without attempting to achieve similar concentrations of each. Naturally, the serum used was 100% serum but Albumax is a powder that had to be dissolved into ddH₂O therefore, it was impossible to achieve 100% dissolved Albumax. Small amounts of Albumax were added to 1.0mL ddH₂O to achieve the highest amount of Albumax in water without reaching the solubility maximum. A final concentration of 20-25% Albumax in 1.0mL of water was achieved which was about 250mg of Albumax. The final Albumax solution was stored in a 4°C for a week until LC-MS. On the day of the LC-MS experiment a new batch of Albumax was made that obtained about the same final concentration. Medium made with Albumax II remains can remain unopened for

weeks before being used to culture parasites therefore, old Albumax II, new Albumax II, and serum metabolites were all compared.

UHPLC-MS of Albumax II and Serum

Despite trying to achieve the highest possible concentration of Albumax II to compare to 100% serum, the levels of metabolites would be drastically different and highest in serum if no other reduction in concentration took place. To try and find the perfect amount of serum to compare to Albumax II, different concentrations of serum and Albumax II were evaluated using reverse-phase ultra-high-performance LC-MS (UHPLC-MS). Serum, new Albumax II, and old Albumax II were all diluted to concentrations of 0%, 20%, 40%, 60%, 80%, 100% with HPLC-grade water for UHPLC-MS. The final sample volume was 100µL. Then 900µL of chilled HPLC-grade methanol containing the internal standard $^{13}\text{C}_4$, $^{15}\text{N}_1$ -aspartate (to control for sample preparation) was added to the samples. Each trial was prepared and analyzed in triplicate technical replicates. Once all 54 samples plus four blanks were prepared, samples were vortexed and then centrifuged for 10 minutes at 15,000rpms at 4°C. The supernatant was transferred to a new tube and dried down using a steady stream of nitrogen to obtain only the metabolites being evaluated without solvent. The metabolite pellet was resuspended in 50µl of HPLC-grade water and spun again to ensure that only liquid and no solid was extracted and injected in to the UHPLC-MS instrument. A total of 58 samples were randomized and run on a Thermo Exactive Plus Orbitrap UHPLC-MS to measure hydrophilic metabolites using a C₁₈ column (Phenomenex

Hydro-RP; catalog no. 00D-4387-B0) and a 25-min gradient of 3% aqueous methanol-15mM acetic acid-10mM tributylamine ion pairing agent and 100% methanol.

Evaluation of UHPLC-MS data

The data obtained from UHPLC-MS had to be converted to a .mzXML file so the data was able to be evaluated using the freeware program MAVEN, a metabolic analysis and visualization engine. The workflow of how to sift through metabolic data is shown in Figure S4. The first step was to run a targeted search on metabolites present in the samples to compared metabolites to a known database. The target search generated hundreds of metabolite matches that had to be assessed by hand for correctness. To determine if the metabolites were correct various indicators were used including the quality of the peak generated (Gaussian shape and signal over noise), proximity to retention time, and observed mass falling within 10ppm of expected m/z. Small speaks with broad bases or peaks with spiky (noisy) appearance were rendered incorrect. Clean strong peaks were kept for further analysis. Once the final list of metabolites 103 metabolites was obtained the levels (peak areas) of each metabolite were compared between, old Albumax II, new Albumax II, and serum at 20%, 40%, 60%, 60%, and 100%. Heat maps were generated using Log₂ mean centered values for hierarchical clustering using a Euclidean distance and ward linkage. All heatmaps were generated in R (<https://www.r-project.org>) with the SupraHex package.

Pharmacologic Intervention

Parasite culture

To prepare 3D7 strain *P. falciparum* parasites for UHPLC-MS the parasites were cultured in a mycoplasma bacteria free hood to prevent possible contamination that could interfere with the metabolite readings. The parasites were checked for mycoplasma weekly using an IntronBio *e-Myco* mycoplasma PCR detection kit. Parasites were cultured in RPMI 1640 with O+ human erythrocytes at 4% hematocrit and kept at 37°C and 5% CO₂. Intraerythrocytic ring-stage parasites were synchronized with 5% sorbitol (Sigma-Aldrich) during three different life cycles. The synchronization method was performed as described by the Llinás Lab (Allman et al., 2016). During synchronization parasites were centrifuged at 1,500 x g for 5 minutes at 25°C, then a 10 X volume of sorbitol was added to the pellet. The parasites were suspended in sorbitol at 37°C for 10 minutes and then was centrifuged at 1,500 x g for 5 minutes. The pellet was resuspended with warm RMPI 1640 media and centrifuged once more. Then parasites were resuspended in 50 mL of media and returned to a flask. The parasites were cultured into three 50mL flasks at 10% trophozoite stage parasites that were 24 hours post invasion. The high parasitemia was needed to obtain about 3×10^8 on the day of magnetic purification before extraction (per experimental condition).

Magnetic Purification

To isolate trophozoite stage parasites from any residual stages present and uninfected RBCs, parasites were run through a magnetic column through a process known as magnetic

purification. The method utilized for magnetic purification was adapted by Erik Allman Ph.D. from the Llinás Lab in 2016 and is detailed in a protocol titled, *Extraction of Drug Treated P. falciparum Trophozoites for LC-MS*. This experiment was performed three separate times and each time in triplicate with 1×10^8 trophozoite parasites each trial.

Drug Treatment

The isolated pure trophozoite parasites were divided into six wells of a 6-well plate. The parasites were given an hour and half to recover from the magnetic purification in the incubator set to 37°C at 5% CO₂. Then, three wells of parasites were treated with 1.7x IC₅₀ of UK-5099, the *P. falciparum* IC₅₀ amount for UK-5099 is 64uM, and the other three wells remained drug free. The concentration 1.7xIC₅₀ was used due to poor compound solubility in water. The parasites returned to the incubator for an additional 2.5 hours. As soon as the 2.5 hours of drug treatment ended, media was aspirated, parasites were transferred to a 2.5mL Eppendorf tube, and quickly centrifuged. The resulting pellet was washed once with ice-cold 1X PBS and repelleted. The washed pellet was then treated with 1.0mL of 90% HPLC-grade methanol (with aspartate standard) to quench metabolism and extract the metabolites.

UHPLC-MS preparation, experimentation, and evaluation

Three methanol blanks were added to empty tubes to control for solvent/processing impurities. All samples were vortexed and centrifuged for 10 minutes at 15,000rpms at 4°C. The supernatant was transferred to a new tube and the samples were dried using a stream of nitrogen gas. Dried-down samples were either stored at -80°C for less a week or immediately resuspended

in 100µl of HPLC-grade water. After resuspension in water, samples were analyzed on UHPLC-MS. Three separate trials were conducted and in each trial, conditions were replicated in triplicate for a total of 9 tubes, including the 3 method blanks, for each trial. Samples were randomized for UHPLC-MS to reduce within-batch variability (Allman et al., 2016). Before the hydrophilic metabolite samples could be analyzed the raw data file had to be converted to a compatible format (.mzXML). Files in the .mzXML format were analyzed using MAVEN and the workflow shown in Figure S4. The three trials were compared with one another using Log₂ fold changes for each UK-5099 sample compared to its respective untreated control. Log₂ fold changes were displayed using an R working environment with the SupraHex package for making heat maps (Allman et al., 2016).

UK-5099 Observation Experiment

Plasmodium falciparum 3D7 parasites were grown in a culture with RPMI 1640 media at 4% hematocrit. Parasites were kept in an incubator at 35°C and 5% CO₂. Parasites were synchronized twice with 5% sorbital and grown to 2% trophozoite parasitemia in a 10mL flask. The flask was then divided into 4 wells of a 6 well plate. To achieve 1% trophozoite parasitemia in each well, 2.5mL of media was added to the 4 wells. Two wells were treated with 1xIC₅₀ UK-5099 (64uM) (Kanaani and Ginsburg., 1992) and two wells remained drug free. An initial slide was taken on day 0. Over the course of 2 life cycles (4 days including drug introduction day), parasites were monitored with blood smears taken at 2 time points every day. Parasites remained under drug treatment and the control parasite's parasitemia was reduced as needed (parasitemia kept under 10%).

Chapter 4

Results

Plasmids generated to knock out the *P. falciparum* *bckdh* gene

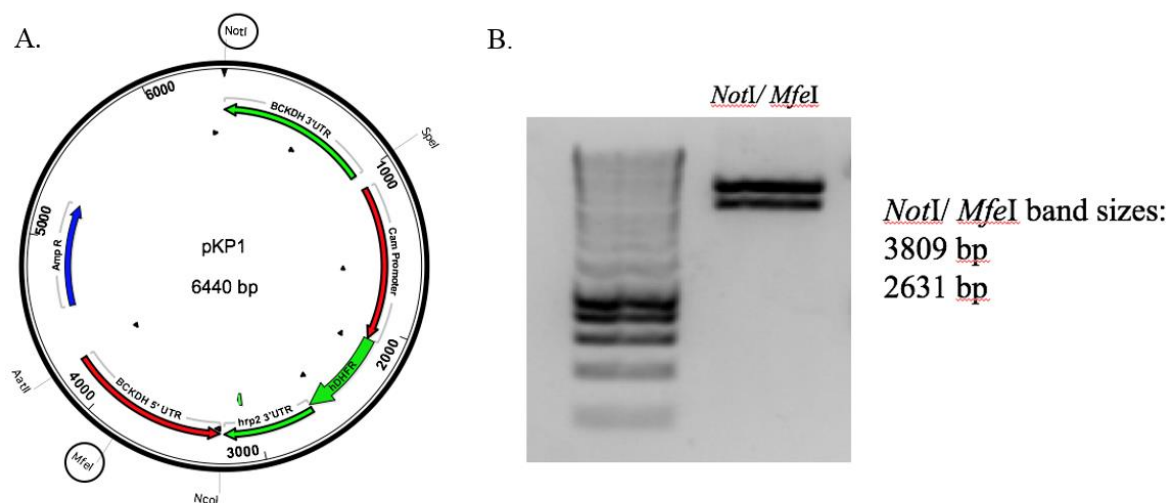


Figure 10 A. The final plasmid, pKP1, with the 5' UTR and 3' UTR inserted into the plasmid. The *NotI* and *MfeI* restriction sites are depicted on the plasmid. B. The diagnostic gel of pKP1 cut with *NotI* and *MfeI*, the top band is 3809bp and the bottom band is 2631bp.

The genetic approach of studying the role of BCKDH in *P. falciparum* involved the generation of *bckdh-e1a* knockout parasites as it was done previously in *P. berghei* and *T. gondii* (Oppenheim et al., 2014). *Pfbckdh* knockout parasites would allow for experimentation with growth assays, metabolic assays, and virulence assays to determine what the function of the enzyme BCKDH is in *P. falciparum*. The beginning of the cloning process for generating *bckdh-e1a* knockout parasites has been completed. The plasmid, pKP1 was generated with both the 5'UTR and 3'UTR homology regions as well as the gene that encodes hDHFR (Figure 10A). This plasmid was transfected into parasites in an attempt to knockout the gene for *bckdh-e1a* from the *P. falciparum* genome via double homologous recombination with the gene for *hDHFR*

in pKP1. To enhance the probability of a double strand break and double homologous recombination, the CRISPR-Cas9 system was also used. The protein Cas9 is an endonuclease that generates a double strand break in a specific location based on the sequence of the guide RNAs used (Lander et al., 2016). For this experiment, guide RNAs were targeted to the middle of the *bckdh* gene. Therefore, once the plasmid is transfected into parasites the Cas9 protein can initiate a double strand break and increase the odds of double homologous recombination with pKP1. The Cas9::*sg-bckdh* plasmid has been made with guide RNAs (sgRNA) targeted specifically to the middle of the *bckdh* gene, as mentioned (Figure 11A). The plasmid, pKP1 was verified using DNA sequencing and restriction enzyme digestion with *Mfe*I and *Not*I (Figure 10B). *Mfe*I and *Not*I cut the plasmid in the middle of the BCKDH 5'UTR, resulting in two fragments with the sizes 3809 and 2631 (Figure 10A,B). The Cas9::*sg-bckdh* plasmid was verified with DNA sequencing (Figure 11B). Most transfections were performed with 100ng of pKP1 and 100ng total of pKP1 and pCas9::*sg-bckdh*; however, no transfected parasites have been generated. Two transfection methods have been repeated many times, the direct electroporation method with parasites and parasite transfection by invasion of DNA-loaded erythrocytes. To try and enhance the probability of generating transfected parasites, selection drug concentrations were reduced and transfections were tried with higher DNA concentrations however, transfected parasites have still not arisen. In the future, these plasmids will continue to be electroporated into parasites to eventually generate a *bckdh-e1 α* -knockout parasite in *P. falciparum*.

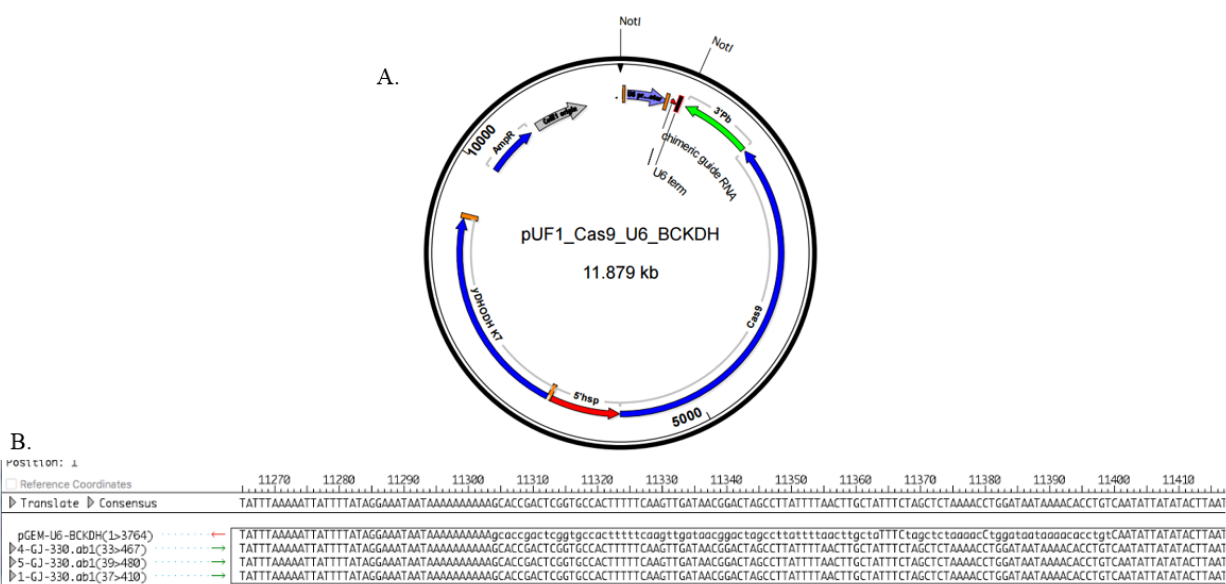


Figure 11 A. The final pUF1_Cas9_U6_BCKDH with the guide RNAs correctly inserted at the *NotI* site. B. The genomic sequencing results of the final pCas9::ss-*bckdh* prove the plasmid is correct. The lower case letters represent the sgRNAs sequence and the absence of red nucleotides implies that no mutation in the pCas9 vector occurred after cloning.

Acetate has no effect on *P. falciparum* growth

We anticipate that the successful knockout of *bckdh* will result in parasites that are struggling to survive due to the absence of sufficient acetyl-CoA levels in the mitochondria (Oppenheim et al., 2014). To prevent *bckdh* knockout parasites from dying from disruptions in metabolism, parasites will be supplemented with sodium acetate (Oppenheim et al., 2014). *P. falciparum* can convert sodium acetate to acetyl-CoA that could be fed into the TCA cycle to overcome the depletion of acetyl-CoA as a result of a *bckdh*-knockout. To determine if NaOAc could be a supplement during future experiments, the effect of adding NaOAc to RPMI 1640 media was studied in asexual parasites. Parasites were split into two groups, those that received media supplemented with NaOAc positive media and those that received conventional media (control). Parasitemia was recorded over the course of three 48-hour life cycles, four days total for both

conditions. Parasites are not able to survive parasitemia levels above 10%, therefore both parasite conditions were split on the same days to prevent lethal parasitemia. However, to compare parasitemia levels between the two conditions the true increase in parasitemia was recorded by multiplying the observed parasitemia by the factor it was reduced by. This increased the ease of comparing the parasitemia of both conditions. As shown in Table 1 and Figure 12, there was no difference in parasite growth between control and NaOAc-treated parasites. A t-test was performed to assess the difference between conditions in the experiment. A p-value of 0.406 was calculated indicating that there is no statistical significance between conditions. This indicates that the successful *bckdh* knockout line can be supplemented with acetate to prevent knockout lethality, without any confounding metabolic issues associated with the supplementation.

Table 1 To compare the effect of sodium acetate on *P. falciparum*, Parasite parasitemia was measured over the course of two life cycles. Results from the t-test show that there is no statistical difference in parasitemia between conditions.

Parasite Condition	T ₀	T _{0.5}	T _{1.0}	T _{1.5}	T _{2.0}	T _{2.5}	T _{3.0}
Control	1.00	1.00	7.796	5.434	34.216	16.949	116.21
NaOAc	1.00	1.00	9.148	8.160	53.100	44.122	99.716

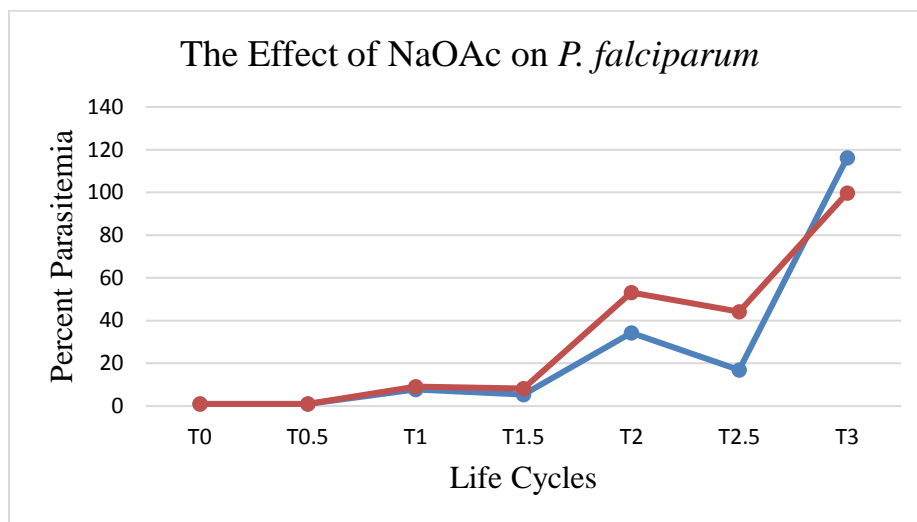


Figure 12 This graph indicates that supplementing parasites with sodium acetate has no effect on parasites. The difference between conditions was found to not be statistically significant after T-test analysis.

Lack of pyruvate derived acetyl-CoA reduces glucose-derived TCA intermediates

The drug UK-5099 inhibits the mitochondrial pyruvate carrier, which transports pyruvate into the mitochondria to be combined with Co-enzymeA (Figure 8A), which forms the acetyl-CoA that feeds into the TCA cycle. BCKDH is assumed to convert pyruvate to acetyl-CoA therefore; the drug UK-5099 should successfully mimic the proposed effect of knocking out BCKDH.

Late-stage trophozoite parasites were purified and treated with UK-5099 for 2.5h at $1.7 \times \text{IC}_{50}$.

The effects of the drug on metabolites were measured via UHPLC-MS. Figure 13B shows that glucose-derived products of the TCA cycle are depleted in the presence of UK-5099. To start.

the drug UK-5099 caused a slight accumulation of pyruvate, which was expected because pyruvate was partially inhibited from entering the mitochondria; therefore, levels would start to build up. In addition to the accumulation of pyruvate, the pyruvate intermediate, phosphoenolpyruvate, also accumulated, altering the levels of downstream metabolites. As a

result of blocking pyruvate from the mitochondria, coenzymeA could not combine with pyruvate to form acetyl-CoA therefore, coenzymeA levels increased to a similar extent of pyruvate. Conversely and as expected, acetyl-CoA levels decreased (Figure 13A). The metabolites, citrate/isocitrate, experienced the greatest depletion because citrate is formed from the combination of acetyl-CoA and oxaloacetate. Acetyl-CoA levels were reduced therefore, citrate/isocitrate was reduced. Citrate and isocitrate had to be measured in one peak because the mass spectrometer did not have the resolution to distinguish the two isomers from each other. The decrease in aconitate, which is the enzyme-bound intermediate in between citrate and isocitrate, reaffirmed the decrease in the combined peak citrate/isocitrate.

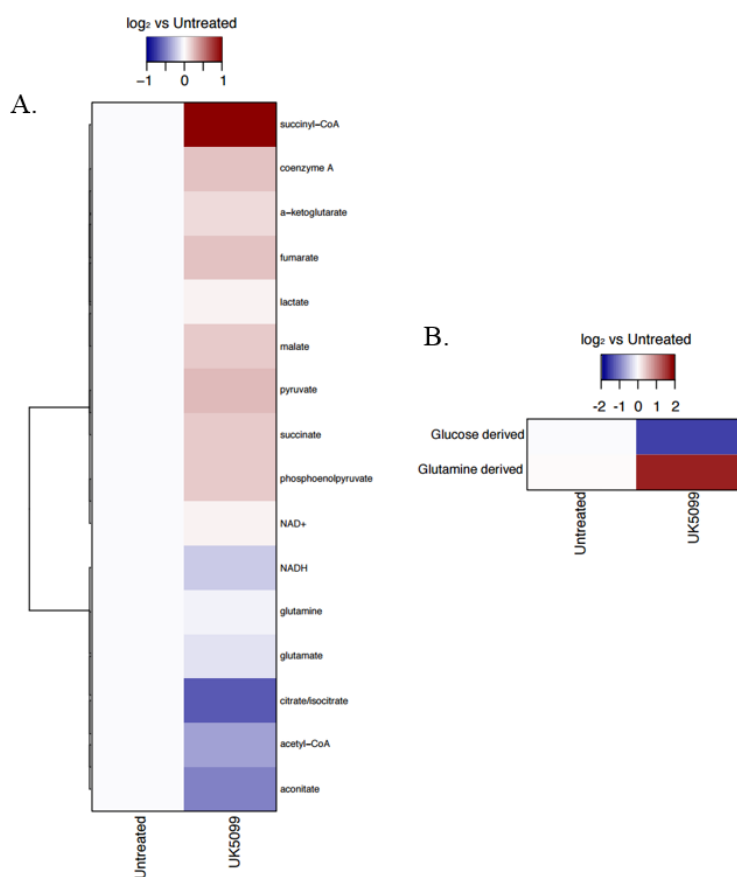


Figure 13 A. This heat map highlights the differences in TCA metabolite levels between UK-5099 and untreated parasites. B. This heat map is a summary of the effect of UK-5099 on glutamine and glucose derived metabolites. Blue represents a depletion while red indicates an accumulation of metabolites.

Glutamine-derived intermediates propel the TCA cycle in the presence of UK-5099

P. falciparum parasites are known to be able to rewire their metabolism to accommodate for environmental and developmental changes (Ke et al., 2015). Therefore, when UK-5099 was added to trophozoite parasites for 2.5 hours it is not surprising that parasites were able to adjust their metabolism to accommodate for the lack of acetyl-CoA entering the TCA cycle. UK-5099 treatment results in a 2-fold depletion of glucose-derived intermediates (Figure 13B) and a 2-fold increase in glutamine-derived TCA cycle intermediates. Glutamine and glucose are the two most important carbon sources for the TCA cycle (Cobbald et al., 2013). Glutamine has been found to be the major carbon supplier for the TCA cycle so it is not surprising that once glucose-derived aerobic respiration was diminished, the glutamine-derived intermediates increased (Ke et al., 2015). Glutamine is converted to glutamate via glutaminase, which is then converted to α -ketoglutarate and fed into the TCA cycle. Glutamine can also enter the TCA cycle through malate via the mitochondrial malate shuttle (Ke et al., 2015). This is supported by Figure 14, where both malate and α -ketoglutarate were more abundant in UK-5099 parasites than untreated trophozoite parasites. In addition to malate and α -ketoglutarate other $\alpha\alpha$ -ketoglutarate downstream intermediates were present in higher concentration such as, fumarate, succinate, and succinyl-CoA. Due to the supplementation of glutamine to propel the TCA cycle in the absence of acetyl-CoA, the change in metabolite fluxes are more significant in the first half of the TCA cycle (acetyl-CoA through citrate/isocitrate) because this portion is more strongly affected by UK-5099. Because of the increased demand for glutamine, glutamine and glutamate were both decreased in UK-5099 treated parasites. Due to the results from the observation experiment, mentioned below, it is assumed that over time glutamine as the sole supplier of the TCA cycle would not be enough to keep the parasites alive.

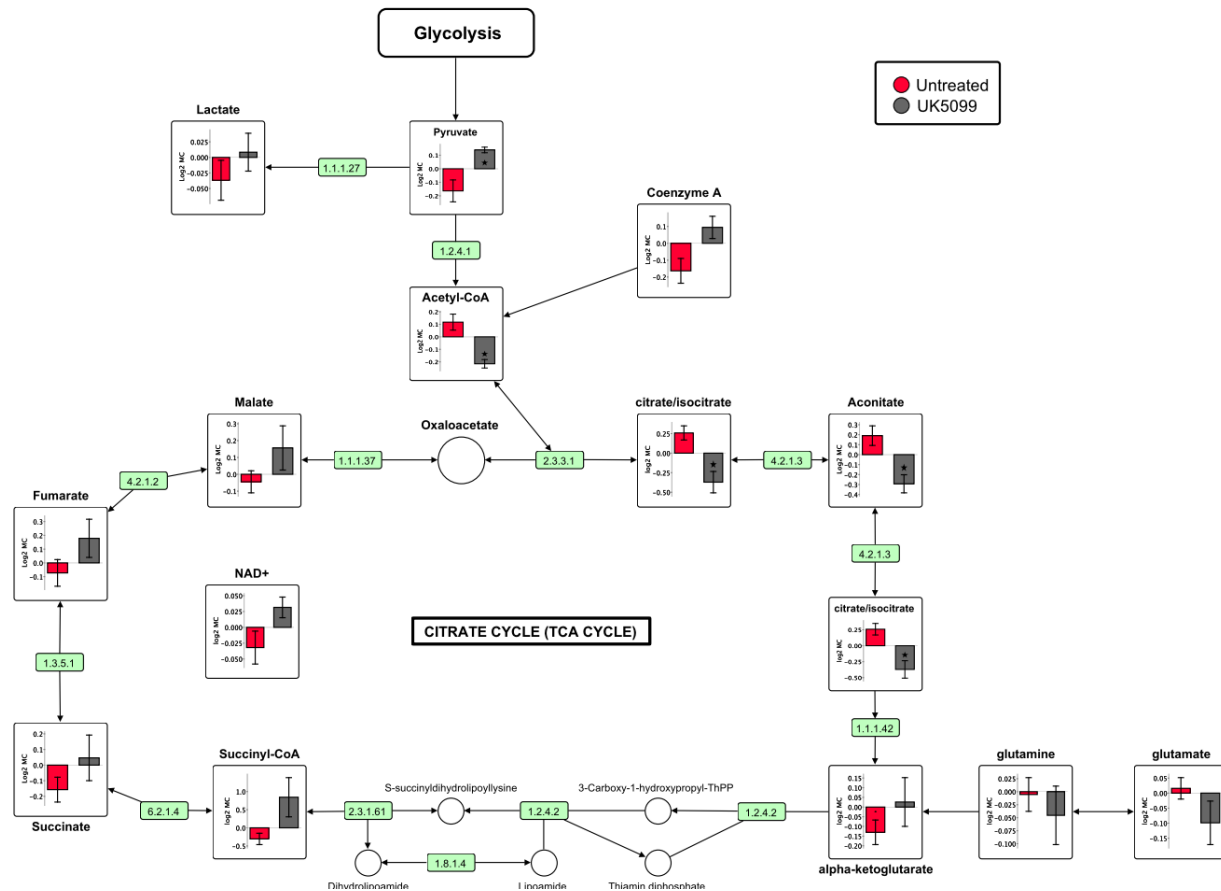


Figure 14 The TCA cycle with the metabolic differences between UK-5099 treated parasites (grey) and untreated parasites (red) generated by log₂ mean centered data. Metabolites with an asterisk are significant (p < 0.05) via the unpaired t-test assuming homoscedastic error. All metabolite plots are standard error from n=3.

UK-5099 causes an increase in aerobic respiration

Although *Plasmodium* spp. convert most of the glucose they salvage to lactate via anaerobic respiration pathways (MacRae et al., 2013), during intraerythrocytic trophozoite and schizont stages there is an increase in aerobic respiration through the TCA cycle. In UK-5099-treated trophozoite parasites there was a slight increase in the level of lactate in parasites (Figure 13A). This increase could either be the result of increased anaerobic respiration as a result of MPC inhibition and pyruvate accumulation or this could be an off-target effect UK-5099. Although

UK-5099 mainly inhibits the MPC, lactate transport can also be inhibited to a lesser extent (Kanaani and Ginsburg, 1992). Therefore, the accumulation of lactate could be caused by off-target inhibition of lactate transport. It is impossible to know what the true cause of increased lactate is without sampling the media to see if there is a lactate excretion phenotype.

UK-5099 causes a decrease in pyrimidine biosynthesis and hemoglobin catabolism

UK-5099-treated parasites experienced an increase in NADP⁺ compared to normal levels. Normally, NADP⁺ is reduced to NADPH through the TCA cycle and then NADH carries electrons to the electron transport chain for pyrimidine biosynthesis to generate cytosine and thymine for DNA replication. UK-5099-treated parasites had an accumulation of NADP⁺ and decrease in NADPH implying that there could have been a decrease in electron transport to the electron transport chain. Overtime, this might have decreased the efficiency of the electron transport chain and pyrimidine biosynthesis. The increases and decreases in metabolites that are important for pyrimidine synthesis such as xanthosine, dTMP, UMP, UDP, GDP, dUMP and many others support the conclusion that UK-5099 and acetyl-CoA depletion could have decreased pyrimidine biosynthesis (Figure 15). dUMP is the metabolite present in the highest concentration in UK-5099 treated parasites signifying that RNA/DNA nucleotide synthesis is not occurring properly, which will lead to a lack of DNA replication and parasite death. In addition to changes in pyrimidine pathway metabolites, peptides such as PEEK, DLS, DLH, PV, VD, and PE are decreased in UK-5099 treated parasites (Figure 15). *P. falciparum* are not able to perform *de novo* synthesis of amino acids therefore, all amino acids except isoleucine, must be obtained through the digestion of hemoglobin from host RBCs (Skinner-Adams et al., 2010). The

reduction in the amount of peptides signifies a decrease in hemoglobin digestion, which is essential for parasites survival. Hemoglobin is digested in a specialized organelle called a digestive vacuole that depends on a pH gradient to stabilize pH at 5.2. Parasites also have neutral aminopeptidases, which are required for hemoglobin digestion and they become inactive at pH less than 6 (Skinner-Adams et al., 2010). It is possible that the buildup in lactate caused pH gradient imbalance within the parasites and decreased the pH in organelles like the digestive vacuole and rendered them inactive. This is a very positive result as a drug that could result in not one inhibition but system-wide dysregulation of many processes, including pyrimidine biosynthesis and hemoglobin catabolism will be more lethal to parasites.

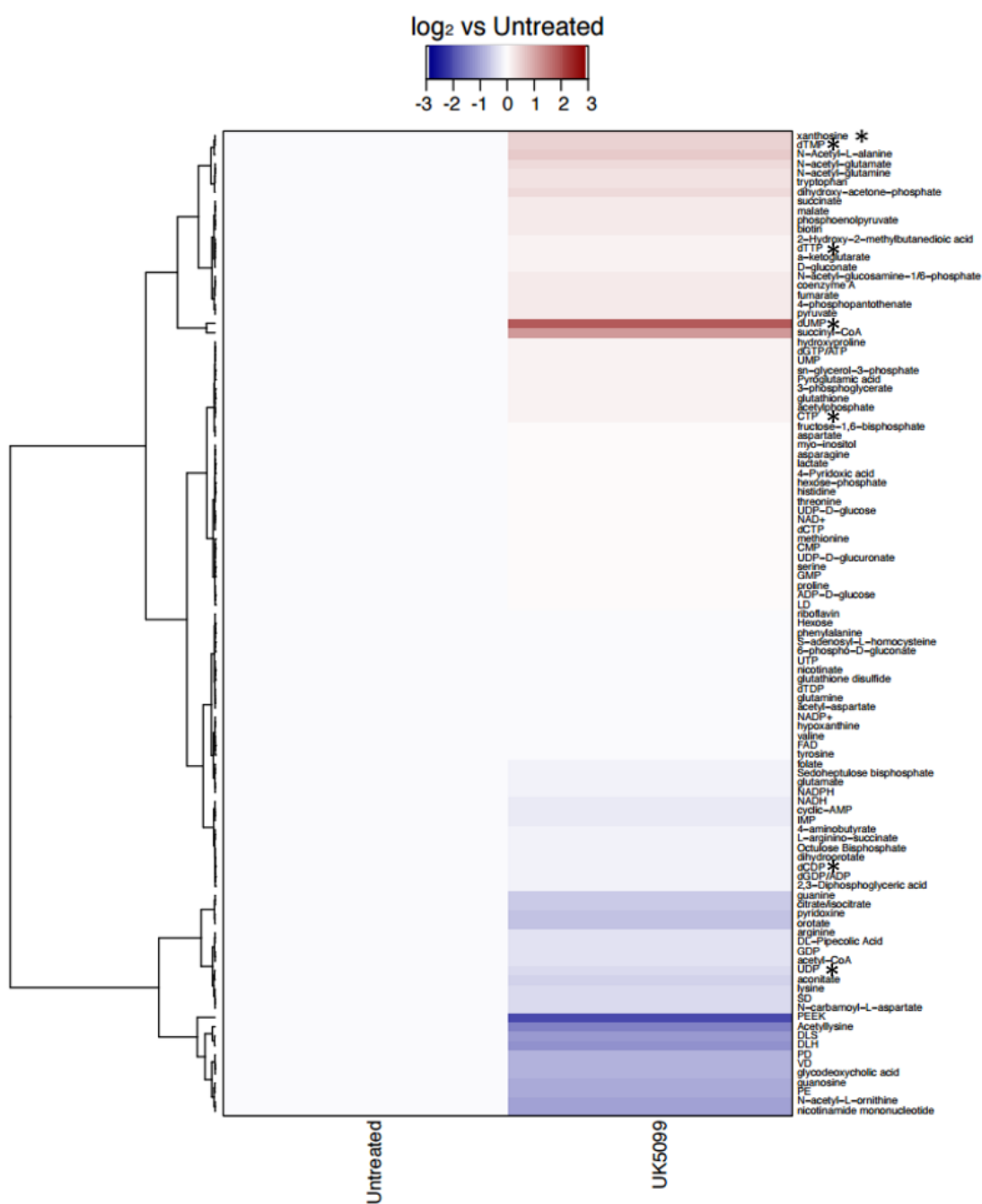


Figure 15 This heat map was generated using Log₂ mean centered data comparing parasites treated with the drug UK-5099 and untreated parasites. Metabolites with * are part of the pyrimidine biosynthesis pathway. Multiple peptides such as PEEK, DLS, DLH, PV, VD, and PE are depleted in UK-5099 parasites. Untreated parasite metabolite levels appear white because those levels were used as the control.

UK-5099 alters the development of *P. falciparum*

To assess how the metabolic disturbances observed affected the physiological and morphological characteristics of the parasite, parasites were cultured in media containing $1 \times \text{IC}_{50}$ (64 μM) of UK-5099 and then observed over the course of 3 days' post drug induction. Figure 16 shows the progression of the effects of UK-5099 on parasites over the course of four total days. Based on the appearance of parasites, the effects of the drug were first noticed after 30 hours. Instead of transitioning from trophozoite staged parasites, which were present at ~19 hours post drug induction, to rings, some parasites began to show signs of death in the trophozoite stage. This is evident by trophozoites beginning to swell and osmophilic bodies clustering within the parasite (Figure 16 30h, b). At this time, the control parasites were rings. Some of the parasites under drug were able to mature correctly to rings (Figure 16 30h, a). Throughout the four total days of observation, parasites that were treated with UK-5099 had a lower parasitemia than the control parasites. This observation supports the assumption that UK-5099 is mainly effecting parasites in the trophozoite stage due to their increased reliance on TCA metabolism and increased demand for DNA replication. UK-5099 might be preventing the maturation of trophozoite parasites to schizonts and ultimately preventing the development of new ring stage parasites. Based on observations of blood smears it appeared that at hour 68 there was a strong difference between the control and drug-treated parasites. While the majority of control parasites were in the ring stage, drug-treated parasites either started to turn into gametocytes (Figure 16 hour 68h, a) or became pyknotic parasites indicting that trophozoite parasites are dying (Kerr et al., 1972). Pyknosis is the process of chromatin condensating in the nucleus. Parasites treated with the drug UK-5099, experienced morphologic changes in structure and composition and were prevented from completing the life cycle, leading to death.

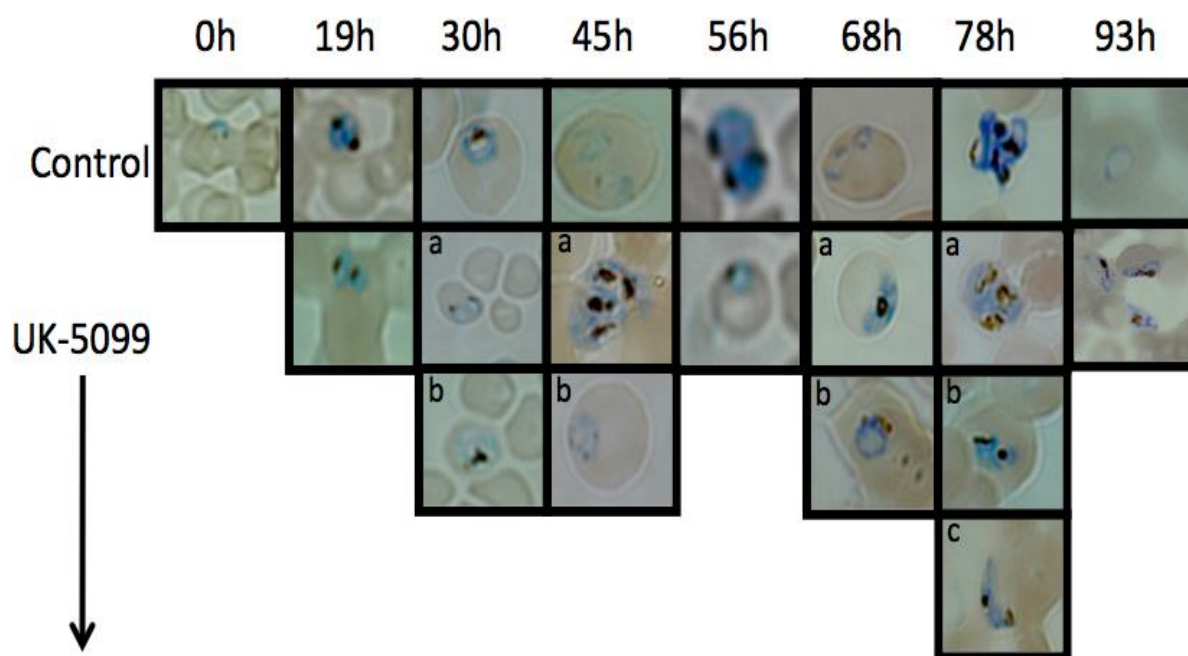


Figure 16 The effect of the drug UK-5099 on the *P. falciparum* life cycle. Parasites treated with drug became unable to develop from trophozoite to ring stage. As the parasites develop in the trophozoite stage, it starts to experience irregular growth patterns. The a, b, and c labels were used when there were multiple stages present or different morphological features in the UK-5099 treated parasites.

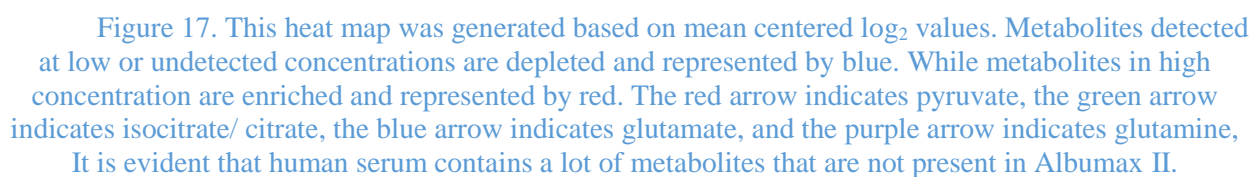
Serum has an abundance of metabolites compared to Albumax II

It is known that parasites that are cultured in Albumax II are unable to produce parasites that can elicit a malaria infection in mosquitoes. To explore the metabolic difference between human serum and Albumax II, UHPLC-MS was used to measure the difference between hydrophilic metabolite concentrations. As Albumax II ages it becomes less beneficial for parasites during culturing, therefore, new and one week-old Albumax II were separately run on UHPLC-MS to observe a possible metabolic difference between them. Serum, old Albumax II, and new Albumax II were prepared at varying concentrations for UHPLC-MS: 20%, 40%, 60%, 80%, and 100%. Originally, Albumax II was run on UHPLC-MS at 0.25%, which is the

concentration used for culturing, however, no hydrophilic metabolites were detected and the concentration had to be increased.

As expected, Serum had a higher amount of hydrophilic metabolites compared to both the old and new Albumax II. Figure 17 represents a heat map comparing the metabolite levels relative to the experimental mean of new Albumax, old Albumax, and serum at designated concentrations. The color blue signifies metabolites that were detected at very low concentrations/ not present. It is important to note that the majority of metabolites that are present in high levels in serum (red) are nonexistent or hardly detectable in all concentrations of Albumax II. There is up to a 15-fold difference between metabolites in Serum in Albumax (Figure 17). Also, the metabolite abundance of serum increases as the concentration increases (ie. light red goes to dark red) while the metabolite abundance of Albumax II does not change between concentrations (Figure 17). This is indicative of metabolites being absent and not just in low levels. Albumax II is most notably deficient in glutamine (blue arrow) and glutamate (purple arrow) (Figure 17), which have been proven to be extremely important for asexual and gametocyte development and could be one of the causes for deficient gametocytes (Ke et al., 2015).

Although serum contains the majority of metabolites, there are two compounds that were much higher in Albumax II, citrate/isocitrate and pyruvate. Citrate/isocitrate (green arrow) and pyruvate (red arrow) were present in excess amounts in Albumax II compared to serum, which contained low levels of both metabolites (Figure 17). The high abundance of citrate/ isocitrate and pyruvate has the potential to inhibit the beginning steps of the canonical TCA cycle. This negative feedback combined with the low levels of glutamine and glutamate have the potential to prevent full maturation of gametocytes by preventing the TCA cycle, which is important for



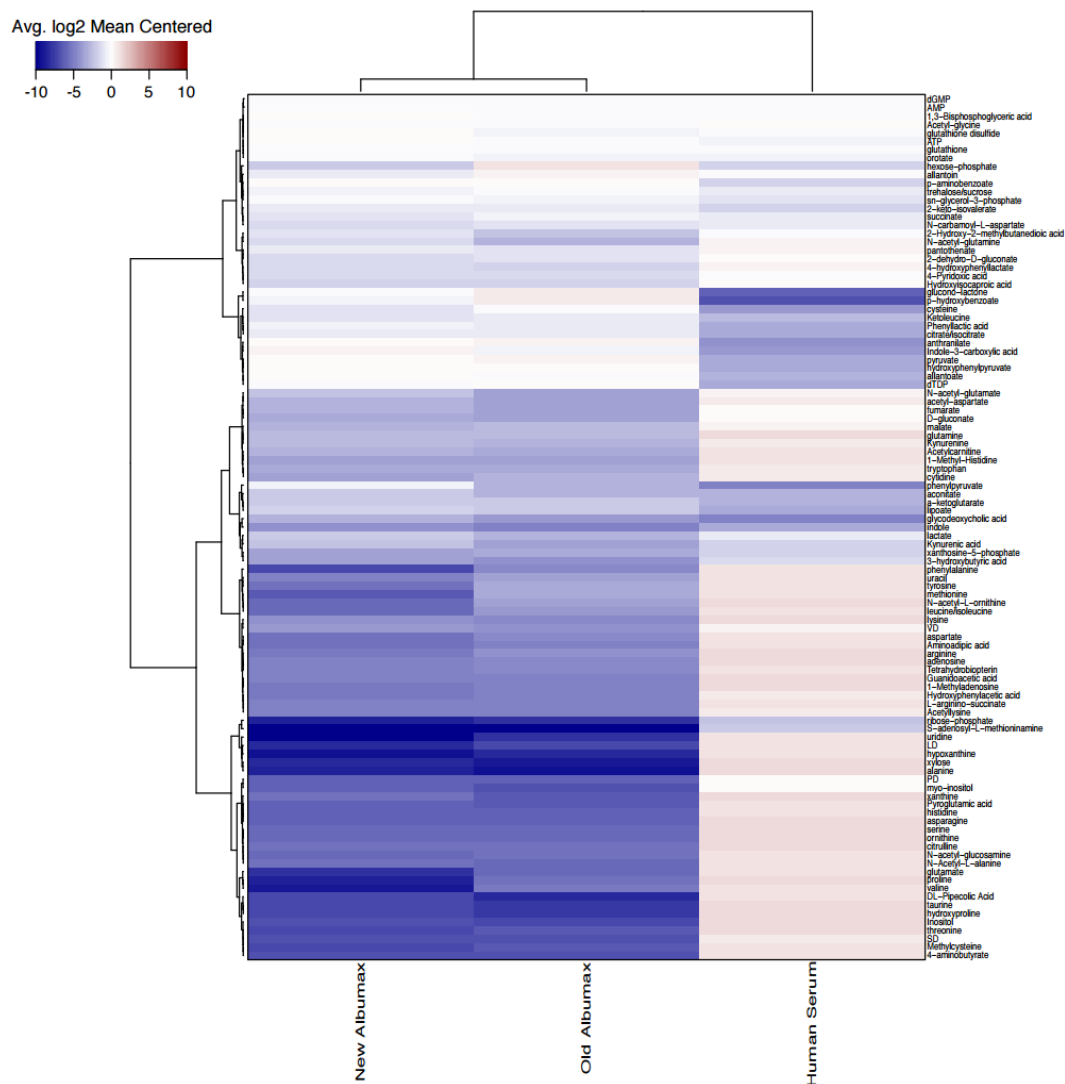


Figure 18 This heat map is a log₂ mean centered heat map that was generated from the average abundance of metabolites across all tested concentration. Once again, Serum has an abundance of metabolites compared to Albumax II. Also, there is very little difference between new and one week-old Albumax II.

Chapter 5

Discussion

Malaria continues to persist as a major global health burden despite multiple attempts to control and eradicate the disease (Cibulskis et al., 2016). Although overall incidence and mortality rates continue to decrease and current malaria drugs are effective, there is a growing fear that parasites are developing resistance again (WHO., 2016). In an attempt to stay ahead of malaria parasite resistance, there is a strong effort for the discovery of new antimalarial targets and a vaccine. The interconnected nature of the parasite's metabolism provides the perfect network to be targeted by antimalarial drugs and many efforts are underway to identify these targets (Plata et al., 2010) (Oppenheim et al., 2014) (Skinner-Adams et al., 2010). Current antimetabolites, such as the antifolates drugs and atovaquone, have been widely successful already, which further proves that parasite metabolism should be explored for new drug targets (Müller and Hyde, 2010).

When the metabolic enzyme BCKDH, which is proposed to be responsible for converting pyruvate from glycolysis to acetyl-CoA, was knocked out in *P. berghei*, parasites experienced growth defects as well as transmission inefficiencies, providing evidence that BCKDH could be a viable drug target (Oppenheim et al., 2014). However, *P. berghei* is not responsible for millions of malaria infections and thousands of deaths annually, *P. falciparum* is. Therefore, to assess whether BCKDH has the same function in *P. falciparum* and if it could be a potential drug target, I further characterized the role of BCKDH in *P. falciparum* through two approaches. The first was a genetic approach that involves the end goal of generating *bckdh* knockout parasites and the second is a combined pharmacology and metabolic approach.

Progress of the Genetic Approach

Significant progress has been made with the genetic approach to studying BCKDH, evident by the generation of both pKP1 and pCas9 final plasmids that will be used to generate *bckdh* knockout parasites. Despite the generation of both plasmids, the cloning project involved a series of difficulties that had to be overcome. From the beginning of the cloning process, I had issues working with the *Plasmodium* genome, which is one of the highest A/T rich genomes to date (Gardner et al., 2002). Before the cloning process started, primers had to be designed with the highest possible concentration of guanine and cytosine to allow for specific binding, which is no easy feat with 90% A/T composition in intergenic regions. Once the primers were designed they were used to extract two intergenic regions on either end of the BCKDH gene, the 5' UTR and the 3' UTR. Generating both the 3' UTR and 5' UTR bands with PCR, involved many protocol manipulations. To start, the primers had very low melting temperatures, which made finding the perfect PCR protocol difficult and PCR often resulted in non-specific or even no products. When the correct 5' UTR was generated and ligated into the pHHT-FCU vector, it would appear that everything was correct until DNA sequencing revealed that recombination events had occurred within the plasmid. After many months of altering PCR protocols and dealing with recombination issues, the 5' UTR was inserted into the vector pHHT-FCU. The next step was to inserting the 3' UTR PCR product into the 5' shuttling vector, which proved itself even more difficult, leading to many attempted troubleshooting techniques. Amplification of the 3' UTR with traditional PCR generated non-specific bands despite alterations to the PCR protocol. Eventually, traditional PCR was abandoned and we switched to Nested PCR, which increased the feasibility of isolating a specific band by using four primers and two rounds of PCR. The first round of Nested PCR amplifies a larger fragment from the genomic DNA and

then that larger fragment is used as a template for a subsequent round of PCR to generate the desired, smaller PCR fragment.

Nested PCR did generate the 3'UTR fragment however, there were still issues with insertion into the 5'shuttling vector. These difficulties could have originated in the size of the final vector being about 9173kb, as larger plasmids become unstable. The New England Biolabs protocol for the Klenow Fill in—a process of blunting the ends by a 3' overhang removal and fill-in of 3' recessed (5' overhang) ends using DNA Polymerase—was used to try and remove a portion of the shuttling vector, although sequencing revealed that the final, smaller plasmid had mutations in the multiple cloning site and could not be used.

The next attempt to reduce the size of the plasmid was slightly more drastic and involved removing the FCU region which enables parasites that have been successfully transfected with the plasmid and undergone double homologous recombination but have not lost the episomal plasmid to be killed in the presence of the drug 5-fluorocytosine. This drug kills all the parasites expressing *fcu* and would also kill parasites who have not undergone double homologous recombination (Braks et al., 2006). The removal of the FCU cassette was successful and the 3' UTR fragment was inserted into the FCU cassette region in one ligation step. Genomic sequencing proved that the final plasmid, pKP1 was correct.

Without the FCU region in the pKP1 vector, there is no way to increase the efficiency of double homologous recombination; therefore, the CRISPR-Cas9 system was used. Guide RNAs were specifically made to target the middle of the *bckdh* gene and then inserted into the Cas9 vector. Once transfected into parasites, the endonuclease Cas9 protein will be expressed and generate a double strand break in the middle of the *bckdh* gene. This double strand break will increase the frequency of double homologues recombination via homologous directed repair with

the pKP1 plasmid (Lander et al., 2016) Transfections with only pKP1 and both pCas9::sg-*bckdh* and pKP1 have been attempted with multiple transfection protocols. The complications with transfections may lie with the stability of the pKP1 vector and long-term success of the cloning project might require generating a new vector. Also, the DNA for transfections might need to be prepared with a new protocol to ensure that all DNA remains intact and DNA concentration is not lost before electroporation occurs. Lastly, it is possible that the enzyme BCKDH is an essential for *P. falciparum* survival, in which case a complete knockout of the gene would not be possible. Therefore, in the future an incomplete knockout can be made. Although only the beginning of the cloning project was completed, the second approach to charactering the role of BCKDH in *P. falciparum*, that used pharmacology and metabolomics revealed many important discoveries about how a knockout of BCKDH would affect *P. falciparum* parasites.

Pharmacology Approach

As an alternative strategy, I investigated the effect of the drug UK-5099 on *P. falciparum*. UK-5099 is the most potent inhibitor of the mitochondrial pyruvate carrier (Kanaani and Ginsburg., 1992) and has the ability to inhibit carrier function at 50nM or less in a rate heart model, which is less than its IC₅₀ amount of 0.064mM in *P. falciparum*. UK-5099 modifies a thiol group on MPC rendering the carrier ineffective and inhibiting pyruvate from entering the mitochondria, resulting acetyl-CoA depletion (McCommis and Finck, 2015). The effects of acetyl-CoA depletion as a result of UK-5099 would elicit a similar response in parasites as a knockout of BCKDH. The results of adding 1.7xIC₅₀ of UK-5099 to trophozoite stage parasites for a duration of 2.5 hours supports this conclusion. When BCKDH was knocked out in *P.*

berghei, parasites had increased levels of pyruvate, fumarate, and malate. Parasites experienced decreases in acetyl-CoA, an acetyl-CoA intermediate, and citrate (Oppenheim et al., 2014). Researchers also observed an increased carbon flux from glutamine and a decrease in glucose-derived TCA metabolites. Asexual knockout parasites experienced diminished ability to grow and remained at low parasitemia. All of these results from *P. berghei bckdh* knockout parasites were observed to some extent with the drug UK-5099, proving that a *bckdh* knockout in *P. falciparum* would likely experience similar alterations.

The drug UK-5099 prevented parasites from generating acetyl-CoA from mitochondrial pyruvate proven by high levels of pyruvate and low levels of acetyl-CoA in UK-5099 treated parasites (Figure 13A). The decrease in acetyl-CoA signifies inhibition of the glucose-feed portion of the TCA cycle. To prevent significant reductions in all TCA metabolites in the absence of glucose feeding into the cycle, glutamine increased its contribution by converting to glutamate, which fed into the TCA cycle as α -ketoglutarate (Figure 13B). The parasites' ability to manipulate its metabolism to account for different environments as well as different developmental stages is believed to be one of the reasons why the parasites are able to be so successful in hosts as well as overcoming drug challenges and developing resistance (Plata et al., 2010) (Cobbold et al., 2013).

For the purpose of this study, metabolites in the second half of the TCA cycle (α -ketoglutarate to malate) were deemed glutamine-derived based off past literature involving isotope labeling experiments of the TCA cycle (Ke et al., 2015). Modifications of the TCA cycle with either inhibitors or enzyme knockouts that prevent glucose-derived carbons, all show a strong preservation of metabolites after α -ketoglutarate by increasing the flux of carbons from glutamine (Ket et al., 2015)(McRae et al., 2013)(Oppenheim et al., 2014). In the presence of UK-

5099, glutamine-derived metabolites experienced a 4-fold increase over glucose derived metabolites (Figure 13A, B), reaffirming past research and stressing asexual staged parasites strong conservation of the latter half of the TCA cycle. The following glutamine-derived metabolites were increased in the presence of UK-5099: α -ketoglutarate, fumarate, malate, succinate, and succinyl-CoA. Although succinyl-CoA appears to be the most abundant metabolite in UK-5099 treated parasites, this does not portray accurate metabolite levels. The first trial had much higher levels of succinyl-CoA compared to the second two, throwing off the total abundance. Variations in drug treatment and sample preparation between UHPLC-MS trials are technical limitations that would need to be addressed through additional studies.

Although, asexual parasites generate all of their ATP from glycolysis and 93% of glucose is excreted as lactate, the TCA cycle still has important functions in *P. falciparum* (MacRae et al., 2013). One of the main purposes of the TCA cycle is to propel *de novo* synthesis of pyrimidines by maintaining the electron transport chain. To drive the conversions of succinate to fumarate, and malate to oxaloacetate in the TCA cycle, electrons are transferred to ubiquinone, which reduces to ubiquinol. Ubiquinol (QH₂) is the electron carrier that reoxidizes the mitochondrial inner-membrane dehydrogenases in the electron transport chain. If asexual parasites are inhibited from reoxidizing inner-membrane dehydrogenases with inhibition of QH₂, such as with the antimalarial atovaquone, asexual parasites will die (Ke et al., 2015). Pyrimidines are vital for DNA replication; which parasites rely heavily on since every parasite gives rise to 16-32 merozoites during the 48-hour life cycle. Without the ability to do pyrimidine biosynthesis, parasites would not be able to replicate and would ultimately die.

Drug treatment with UK-5099 caused many of the metabolites involved in pyrimidine biosynthesis to be present at either high or low levels compared to untreated parasites. The

metabolite dUMP was the most abundant metabolite in UK-5099 treated parasites (Figure 15). Changes in dUMP levels signify issues with DNA replication and more specifically thiamine synthesis, which is one of the main nucleotides used to synthesize *P. falciparum*'s A/T rich genome. Thymidylate synthetase converts dUMP to dTMP, which was also increased in UK-5099-treated parasites but to a lesser extent (Figure 15). Aspartate, which is involved in the first committed step of pyrimidine biosynthesis, was also present at a higher concentration in UK-5099 parasites. This either signifies that with treatment of UK-5099 there is an increased need for pyrimidines so aspartate must be more abundant or it means that due to the TCA cycle functioning at a lesser extent and decreases in NADPH (Figure 13A) steps in the pyrimidine biosynthesis pathway are being inhibited leading to a buildup of metabolites. The metabolite UTP is the precursor of the rate-limiting step of pyrimidine biosynthesis (UTP converting to CTP), and it was decreased from normal levels in UK-5099-treated parasites. If the rate-limiting step is unable to be completed because of a lack of starting compound, then future end products (pyrimidines) will not be made and asexual parasites will begin to die (Zhao and Hong., 2009). In addition to decreases in pyrimidine biosynthesis, there were significant decreases in the peptides PEEK, DLS, DLH, DV, VD, and PE in UK-5099 treated parasites (Figure 15). Because peptides are made from amino acids, this implies that hemoglobin catabolism was not functioning correctly under the effects of UK-5099 (Berg et al., 2002). A plausible explanation is that the accumulation of lactate decreased the pH within the parasite, resulting in the inhibition of the pH-sensitive hemoglobin catabolism processes (Skinner-Adams et al., 2010).

Evidence that UK-5099 caused dysregulation and inhibition of pyrimidine biosynthesis that resulted in asexual parasites dying is supported by the observation experiment with UK-5099 (Figure 16). Parasites under drug treatment began to die after one day, evident by pyknotic

trophozoites and the beginnings of gametocytogenesis (Figure 16). Also the parasitemia of UK-5099 treated parasites remained much lower than untreated parasites indicating that trophozoites were not able to complete the life cycle. Trophozoites and schizonts are more metabolically demanding compared to other asexual stages. BCKDH transcriptional levels are 5 times higher in late trophozoite parasites and 4 times higher in schizonts compared to levels in ring and early trophozoite parasites (PlasmoBd). This supports the conclusion that trophozoites and schizonts were more strongly affected by UK-5099 (Figure 16). Trophozoites showed the most evidence of death, which follows the knowledge that they require more BCKDH activity and rely more on pyrimidine biosynthesis to undergo DNA replication so new parasites can form (Cassera et al., 2011). In previous experiments with TCA metabolism enzyme knockouts or inhibitors, very little effects were observed in asexual *P. falciparum* parasites. In this experiment, however, asexual parasites had growth defects and started to die after four days of UK-5099 treatment; therefore, asexual parasites may not be able to survive solely on a glutamine-derived TCA cycle and could be susceptible to drugs targeting early enzymes in the glucose-derived portion of the TCA cycle (MacRae et al., 2013)(Ke et al., 2015).

Pharmacology Limitations

It should be noted that all of these observations were noticed at only $1 \times \text{IC}_{50}$ amount of UK-5099. Most drug experiments in the lab are done at $10 \times \text{IC}_{50}$. Using a much higher IC_{50} amount would make the drug effect more pronounced; however, UK-5099 has poor solubility in DMSO, making high IC_{50} levels hard to obtain. Experimentation with other laboratory solvents could overcome this constraint. Observation experiments with UK-5099 should also be repeated

on gametocytes since gametocytes were not able to fully develop in *P. berghei bckdh* knockout parasites. Compared to all asexual parasites, gametocytes use TCA metabolism more and only excrete 80% of glucose in the form of lactate compared to 90% in asexual parasites (MacRae et al., 2013). BCKDH transcriptional levels are also 3x higher in stage V gametocytes compared to ring and early trophozoite parasites (PlasmoDB). Therefore, the UK-5099 observation experiment should also be done on gametocytes to observe how they respond to the drug. It is assumed that there would be a decrease in the number of gametocytes that are able to reach full maturation, a decrease in the ability for sexuals to mature, and a decrease in transmission to mosquitoes as seen in the *P.berghei bckdh* knockout parasites (Oppenheim et al., 2014).

In addition to modifications that need to be made to the UK-5099 observation experiment, the metabolomics experiment with UK-5099 should also be done with higher IC_{50} levels. UK-5099 was added to trophozoite stage parasites at $1.7 \times IC_{50}$. A higher IC_{50} level will give a better representation as to how the parasites are responding to the drug and will provide clearer indications to which metabolites are increased and decreased under UK-5099. It would also be beneficial to increase the parasites exposure to the drug from 2.5 hours to longer time periods. Running a time course over 5 different exposure times would allow for the evaluation of how the drug is affecting parasites over an extended period of time and how the parasites would adapt to the drug pressure (Srinivas and Puri., 2002). The metabolic experiments should also be repeated with glutamine, glucose, acetate, and pyruvate isotope labeling (Oppenheim et al., 2014). Labeling with glutamine and glucose will allow for better interpretation as to how glutamine carbons and glucose carbons are feeding into the TCA cycle after the effects of UK-5099. We would expect to see more carbons labeled with glutamine-derived isotope label than with glucose isotope label in UK-5099 treated parasites. These results would also be comparable

to Oppenheim's experiment, which would allow the ability to analyze the similarity. If the results are highly similar, this could strengthen the thought that BCKDH has the same function in *P. falciparum* as *P. berghei* and that BCKDH or the surrounding pathways could serve as a potential drug target. Isotope labeling with pyruvate would provide the ability to analyze how effective MPC inhibition is. If UK-5099 completely inhibits the MPC, there would be no metabolites in the TCA cycle with pyruvate label. Labeling with acetate would show how much of acetyl-CoA generation is coming from acetate in the absence of the ability to be generated from pyruvate. This would provide insight into how parasites reconstruct their metabolism in glucose-deficient environments, which often occurs in hypoglycemia patients who have severe malaria (Planche et al., 2015).

It should be noted that despite its ability to inhibit growth in asexual parasites, UK-5099 cannot currently be used as an antimalarial drug for many reasons. The drug has only been produced for research use and must be dissolved in large amounts of DMSO (Sigma-Aldrich). Also, humans and *Plasmodium* spp. likely have similar mitochondrial pyruvate carriers and would both be targeted by UK-5099 (McCommis and Finck., 2015). This would cause human cells, evident by the effect in rat hearts, to die extremely rapidly with the depletion of mitochondrial pyruvate, although therapeutic uses for UK-5099 are currently being explored for a variety of diseases (Zhong et al., 2015) (Patterson et al., 2014). Also, there are examples of successful antimalarials that have shared targets between host and parasite (Nixon et al., 2013). Therefore, it is possible that if a selective and potent MPC inhibitor for *Plasmodium* is found, which would require large drug screenings, it could be a potential antimalarial.

Future Directions

While there are a lot of improvements that could be done to both UK-5099 pharmacologic experiments to increase the accuracy and validity of the results, the true assessment on the role of BCKDH in *P. falciparum* parasites will have to come from assays on *bckdh* knockout parasites. Once a *bckdh* knockout parasite can be generated the same experiments that were performed on parasites with UK-5099 will be repeated as well as all the improvements mentioned. More comprehensive experiments will also need to be done such as gametocyte development into oocysts in mosquitoes. *bckdh* knockout *P. berghei* mutants produced considerably less oocytes per infected mosquito midgut. Oocytes also stopped growing at day 7 and were unable to undergo sporogony (Oppenheim et al., 2014). The test of whether BCKDH can be an antimalarial drug target will come from its ability to inhibit sporogony in *P. falciparum* and stop the transmission cycle between humans and mosquitoes.

Serum vs Albumax II

An in-depth analysis of the metabolic differences between human serum and Albumax II shed some light on the importance of the TCA cycle for gametocyte parasites. Serum is derived from O+ human donors and Albumax II is a bovine-serum substitute commonly used to circumvent the inconsistencies and expenses of human serum. However, gametocytes grown in Albumax II are unable to generate an infection and mature into oocytes in mosquitoes. UK-5099 experiments as well as past experiments demonstrate the importance of a functioning metabolism for all stages of parasite development (Ke et al., 2015) (Oppenheim et al., 2014). Therefore, the

difference between Albumax II and serum may be rooted in the affect the mediums have on parasite metabolism.

Overall, serum had an abundance of metabolites compared to Albumax II, which could provide the gametocytes with more metabolites needed for growth. More specifically, serum had high levels of glutamate and glutamine, two metabolites that have been proven to be extremely important for parasite growth. Not only is the TCA cycle mainly supplied through glutamine over glucose, but also it is also extremely important for gametocytogenesis (Cobbold et al., 2013). When KGDH, an enzyme that controls the first committed step of glutamine entering the TCA cycle, was knocked out in gametocytes, they were not able to produce oocysts in mosquitoes (Ke et al., 2015). A similar situation could be occurring in gametocytes cultured in Albumax II, as they are unable to become infectious to mosquitos. Despite having an absence of most metabolites, Albumax II has a massive abundance of citrate compared to the low citrate levels in serum (Figure 17). Citrate is mainly formed from the combination of acetyl-CoA and oxaloacetate through the enzyme citrate synthase (Wiegand and Remington., 1986). Citrate synthase is an irreversible enzyme, naturally making it a major control point of the TCA cycle and its function under high regulation. To start, citrate synthase is inhibited by high levels of citrate (Williamson, 1967). Therefore, the high levels of citrate originating not in the parasite but in the Albumax II supplemented media could result in negative feedback that inhibits the parasite's citrate synthase and subsequently the TCA cycle. Normally, high levels of citrate indicate that TCA metabolites are abundant and glucose degradation should be inhibited by inactivating citrate synthase (Berg et al., 2002).

The major site of glycolysis regulation is phosphofructokinase which is another irreversible and regulatory enzyme. It is responsible for converting fructose 6-phosphate to

fructose 1,6 biphosphate (Berg et al., 2002). In mammalian cells, phosphofructokinase is inhibited by citrate in the form of high ATP; however, current research indicates that *P. falciparum* generate all their ATP from glycolysis and not the electron transport chain because they are missing critical subunits of highly conserved ATPase (Praveen et al, 2011). Therefore, high levels of citrate are not synonymous with high ATP in *Plasmodium*. Although, the non-physiological abundance of citrate could still cause inhibition of phosphofructokinase, resulting in inhibition of glycolysis. In *P. falciparum* citrate can also be derived from phosphophenolpyruvate (PEP) by PEP carboxylase (PEPC), which produces oxaloacetate that feeds into the TCA cycle the step before citrate formation (Cobbold et al., 2013). Further experimentation will have to be done to determine the effect of high levels of citrate on glycolysis regulation and the alternative pathways of citrate formation.

The high levels of citrate in Albumax II might cause negative feedback on glycolysis through inhibition of phosphofructokinase, the beginning steps of the TCA cycle through inhibition of citrate synthase, and also from the alternative pathways such as PEPC. Without the glucose-derived portion of the TCA cycle, the parasites would have to increase the carbon input from glutamate/glutamine; however, Albumax II is deficient in these compounds. The parasites' need for these compounds might be so demanding that without media that has glutamate and glutamine, the parasite could fail to keep up with the metabolic demand. This could cause gametocytes grown in Albumax II to arrest and oocysts to fail to develop in mosquitoes.

It might be assumed that starting at a max concentration of 25%, while serum started at a 100% concentration, hindered the total Albumax II concentration, but this is not the source of Albumax's lack of metabolites. Theoretically, the samples of 100% Albumax II and 20% serum should be equal and when these concentrations are compared, the general trend remains the same

(Figure 17). Also, during culturing the media only contains 0.25% Albumax II which is 100x less than the concentration used for this experiment. Therefore, the lack of metabolites at 25% Albumax II must indicate an extreme lack of metabolites in the 0.25% Albumax II used for culturing. Before definitive conclusions can be made, further investigation into not only target hydrophilic metabolites but also untargeted hydrophilic metabolites and hydrophobic metabolites must be done. If they reveal similar results, parasites should be grown in Albumax II with supplementation of glutamate, to assess whether they are able to overcome the citrate abundance and mature to become infectious to mosquitoes. Also gametocyte metabolism should be probed in the presence of serum and Albumax II to make an accurate comparison. All of these experiments could further provide insight into which metabolites are vital for gametocyte development.

Chapter 6

Conclusion

The examination of BCKDH using the combination of pharmacology and metabolomics revealed more insight into how parasites respond to acetyl-CoA deficient environments. Metabolic experiments on trophozoite stage parasites generated a metabolic pattern that has been affirmed by previous literature. Glucose-derived TCA metabolites were depleted without mitochondrial acetyl-CoA and as a result the carbon flux from glutamine into the TCA cycle at α -ketoglutarate increased by 2-fold. This is what would be expected of *bckdh*-knockout parasites, as pyruvate would not be converted acetyl-CoA. A reduction in the acetyl-CoA pool and an increase in pyruvate validates that UK-5099 does have a similar role as knocking out *bckdh*.

UK-5099 treated parasites experienced drastic changes in metabolites associated with pyrimidine biosynthesis, which is vital for asexual replication and the ability of parasites to maintain an infection in hosts. This supports the conclusion that inhibiting acetyl-CoA formation in the mitochondria could have the ability to inhibit the growth and development of asexual parasites. This outcome was observed with preliminary observation studies on asexual parasites, in which parasitemia was significantly reduced and most parasites visibly began to show signs of death. Under the drug UK-5099 the parasites also experienced dysregulation that extended beyond their TCA metabolism and to hemoglobin catabolism and pyrimidine biosynthesis.

The importance of *P. falciparum* maintaining their TCA cycle and regulating both glucose-derived and glutamine-derived carbon sources, could be the reason why gametocytes grown in Albumax II are not able to develop into infections in mosquitoes, like those grown in serum. It is possible that Albumax II inhibits the glucose-derived portion of the TCA cycle with its abundance of citrate and then the lack of glutamine and glutamate supply prevents the

parasites from fueling the TCA cycle with glutamine-derived carbons. This is a method commonly used in parasites to ensure that the important functions of the TCA cycle remain preserved, as reaffirmed by the UK-5099 metabolic experiments.

Although more assessments must be done to confirm the role of BCKDH and acetyl-CoA formation in *P. falciparum*, the experiments in this thesis prove how important the formation of acetyl-CoA is for parasites survival. The beginning of the TCA cycle, where BCKDH functions, has the ability to be a strong antimalarial target because it has the potential to inhibit the growth of both asexual and sexual stage parasites. This would prevent the symptoms and the transmission of malaria. Also, BCKDH regulation is still unknown and if it is found to be post-translationally modified then there might be an inhibitor that could block the enzyme from being active. If drugs targeting acetyl-CoA production are developed, I propose also treating patients with atovaquone, to ensure that the TCA cycle is completely inhibited and all parasite stages are killed. Using a combination of drugs that target different metabolic processes will slow parasite resistance to both drugs.

Appendix

Supplemental Figures

Supplemental Figure 1: Alignment of BLAST results between human BCKDH and *P.*

falciparum BCKDH

Range 1: 44 to 429 Graphics				▼ Next Match ▲ Previous Match	
Score	Expect	Method	Identities	Positives	Gaps
305 bits(781)	2e-104	Compositional matrix adjust.	161/389(41%)	232/389(59%)	5/389(1%)
Query 59	AEFIDKLEFIQPNVISGIPIYRVMDRQGQIINPSEDPHLPKEKVLKLYKSMTLLNTMDRI				118
Sbjct 44	+EF +L+ + N + +PIYR++D G +++ E P E+VLK+YK M + D I				100
Query 119	LYESQRQGRISFYMTNYGEEGTHVGSAAALDNTDLVFGQYREAGVLMYRDYPLELFMAQC				178
Sbjct 101	Y QRQGRISFY+ N GEEG G AL D ++ QYRE GVL+ R + + Q				160
Query 179	YGNISDLGKGRQMPVHYGCKERHFVTISSPLATQIPQAVGAAYAANKRANANRVVICYFGE				238
Sbjct 161	+G D GKGRQM + Y K+ + TI++PL +Q+ A G YA K N V + Y G+				220
Query 239	GAASEGDAHAGFNFAATLECPPIFFCRNNGYAISTPTSEQYRGDGIARGPGYGIMSIRV				298
Sbjct 221	G++SEGD +A NFA+ + +F C+NN YAIST +QYRGDGI R GI SIRV				280
Query 299	DGNDVFAVYNATKEARRRAVENQPFLEAMTYRIGHHSTSDSSAYRSVDEVNYWDKQD				358
Sbjct 281	DGNDLFAVYATKLRDICIQESKPVFIEFMSYRYGHHSTSDSSLYRPKEENAWRQEG				340
Query 359	-HPISRLRHLYLLSQGWDEEQEKAWRKQSRKVMFAFEQAERKPKPN-PNLLFSDVYQEM				416
Sbjct 341	HPISR+ YL ++ + E++++ RK + V++ ++ E + N LF DVY E				400
Query 417	PAQLRKQESLARHLQTYGEHYPLDHFDDK 445				
Sbjct 401	+++Q+E+ + + +Y F++				429

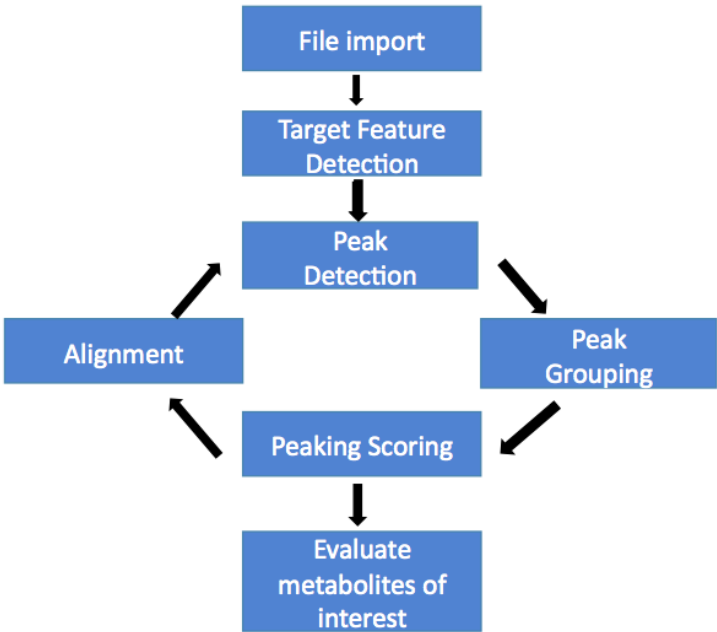
Supplemental Figure 2: The primers used to generate the pKP1 plasmid

Primer Name	Sequence
5' UTR F (AatII)	GAGAGAGACGTGatacacataagtgttagcaataattaagggtta
5' UTR GC (NcoI)	ACACACCATGGGTAAGTATTTCTGAACAATATTTCTCAT
3' UTR F2 (HpaI)	GAGAGAGTTAACTtggttggtggattatttgaagatg
3' UTR GC2 (SacII)	ACACACCGCGGgtattttaatactgaataacctgagtttcc
3' UTR F (SpeI)	GAGAGAACTAGTATGATACATCAAAATTTGAGCGATGA
3' UTR GC (NotI)	ACACAGCGGCCGCGCGGTAAAATTGGGAAAACAGAAATGACTCC

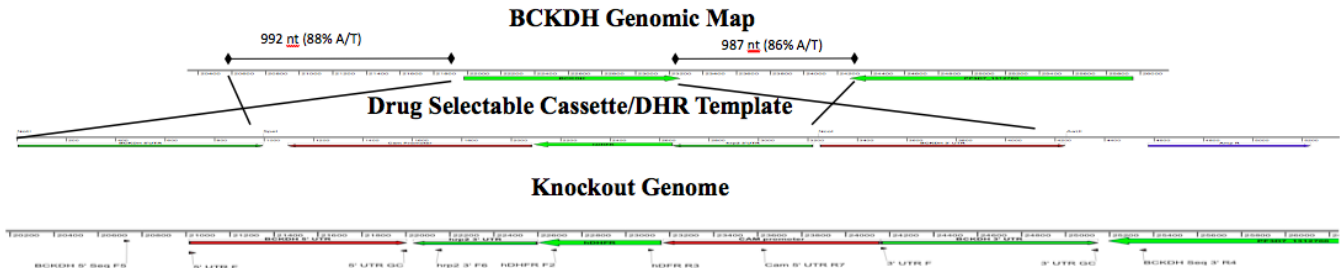
Supplemental Figure 3: The guide RNAs used to target BCKDH in the CRISPR-Cas9 system

Guide RNA	Sequence
BCKDH gRNA F (BbsI)	attgACAGGTGTTTTATTATCCAG
BCKDH gRNA GC (BbsI)	aaacCTGGATAATAAAACACCTGT

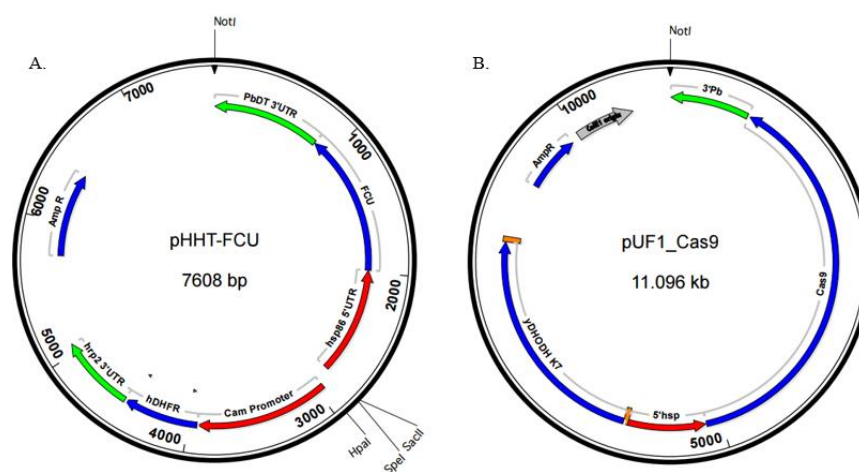
Supplemental Figure 3: The workflow used for evaluating metabolomic data in this thesis



Supplemental Figure 4: The cloning scheme for knocking out BCKDH



Supplemental Figure 5: The original pHHT-FCU vector and pUF_Cas9 vector used for cloning



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Academic Vita of Kayla Paulosky

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Education

Pennsylvania State University, Schreyer Honors College
University Park, PA
BS, Immunology and Infectious Disease, Global Health Minor
Universidad Pablo De Olavide, Sevilla, Spain
Spring 2016

Research

Llinás Laboratory: Penn State Biochemistry and Molecular Biology Dept Aug 2014-present
Undergraduate Researcher

- Research the metabolism of *Plasmodium falciparum*, the parasite that causes malaria, to determine the role of branched chain ketoacid dehydrogenase (BCKDH) in *P. falciparum*.
- Experience with culturing and running experiments with live parasite cultures.

Center for Injury Research and Prevention: Children's Hospital of Philadelphia Summer 2016
Student Researcher

- Team member on a retrospective study evaluating the risk of multiple concussions in young children.

Grants and Scholarships

ECoS Undergraduate Research Support Competition Grant for \$1,000 Fall 2014- Spring 2015

- Project: *Determining the Role of the Branched Chain Ketoacid Dehydrogenase (BCKDH) in P. falciparum.*

Erikson Discovery Grant for \$3,500 Summer 2015

- Project: *Determining the Role of the Branched Chain Ketoacid Dehydrogenase (BCKDH) in P. falciparum.*

Rosemarie C. and Howard R. Peiffer Scholarship for \$2,000 Fall 2014-Spring 2016

Leadership

Empower Orphans Nonprofit Penn State Chapter
College Relations Chair Summer 2016- present

- Constructed an Empower Orphans Chapter Start Up brochure for universities interested in affiliating with a non-profit that has raised over 1 million for orphan children abroad.
- Manage the expansion of Empower Orphans to new universities.
- Created and participated in educational events for elementary school children about service project involvement.
- Assisted in raising over \$600 in one semester for children in need.

Mid-State Literacy Council

Center County, PA

Tutor

Fall 2015

- Organized lesson plans and taught English to two ESL adults for a total of 4 hours every week.
- Prepared one student for the TOEFL.

Global Medical Brigades

Penn State Chapter

Member

Aug 2013-May

2014

- Traveled to Panama over spring break in 2014 to set up temporary clinics with two doctors and a dentist in two rural communities.
- Worked in various departments of the clinic: patient vital intake, pharmacy, dentist, and physician consulting.

Penn State Dance Marathon

Penn State University

Volunteer/ Committee member

Aug 2013-present

- Contributed to the fundraising efforts of the biggest student-run philanthropy organization in the United States, which has raised over \$127 million for children and families affected by pediatric cancer.
- Member of the Production committee. Responsible for filming THON events throughout the year as well as THON weekend. Produced promotional and educational videos with a strict time frame that were viewed by thousands of people.
- Member of the Dancer Relations committee. Responsible for the health and well-being of 2 out of 700 dancers that dance for 46 hours without sitting or sleeping. Created and implemented events that provide relief to dancers as well the THON families during THON weekend.

Work Experience

Organic Chemistry Instrument Room

Penn State Chemistry Dept.

Undergraduate TA

Fall 2015-present

- Instruct students how to analyze their NMR, IR, and GC-MS data, as well as operate the instruments.
- Monitor students as they work with the instruments.

ERMI

Havertown, PA

Independent Contracto

Summer 2016

Instructed and set up patients on end range motion devices to facilitate their