Published in final edited form as:

ACS Infect Dis. 2016 December 09; 2(12): 903–916. doi:10.1021/acsinfecdis.6b00101.

A Novel Methodology for Bioenergetic Analysis of *Plasmodium falciparum* Reveals a Glucose-Regulated Metabolic Shift and Enables Mode of Action Analyses of Mitochondrial Inhibitors

Tomoyo Sakata-Kato and Dyann F. Wirth*

Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, Massachusetts 02115, United States

Abstract

Given that resistance to all drugs in clinical use has arisen, discovery of new antimalarial drug targets is eagerly anticipated. The *Plasmodium* mitochondrion has been considered a promising drug target largely based on its significant divergence from the host organelle as well as its involvement in ATP production and pyrimidine biosynthesis. However, the functions of *Plasmodium* mitochondrial protein complexes and associated metabolic pathways are not fully characterized. Here, we report the development of novel and robust bioenergetic assay protocols for *Plasmodium falciparum* asexual parasites utilizing a Seahorse Bioscience XFe24 Extracellular Flux Analyzer. These protocols allowed us to simultaneously assess the direct effects of metabolites and inhibitors on mitochondrial respiration and glycolytic activity in real-time with the readout of oxygen consumption rate and extracellular acidification rate. Using saponin-freed parasites at the schizont stage, we found that succinate, malate, glycerol-3-phosphate, and glutamate, but not pyruvate, were able to increase the oxygen consumption rate and that glycerol-3-phosphate dehydrogenase had the largest potential as an electron donor among tested mitochondrial dehydrogenases. Furthermore, we revealed the presence of a glucose-regulated metabolic shift between oxidative phosphorylation and glycolysis. We measured proton leak and reserve capacity and found bioenergetic evidence for oxidative phosphorylation in erythrocytic stage parasites but at a level much lower than that observed in mammalian cells. Lastly, we developed an assay platform for target identification and mode of action studies of mitochondria-targeting antimalarials. This study provides new insights into the bioenergetics and metabolomics of the *Plasmodium* mitochondria.

Abstract

*Corresponding Author: Tel.: (617) 432-1563. Fax: (617) 432-4766. dfwirth@hsph.harvard.edu.

Author Contributions

T.S.K. conducted all experiments, analyzed the results, and wrote most of the paper. D.F.W. conceived the idea for the project and wrote the paper with T.S.K.

The authors declare no competing financial interest.

Keywords

malaria; *Plasmodium falciparum*; mitochondria; bioenergetics; mitochondrial inhibitors; mode of action

Protozoan parasites of the genus *Plasmodium* are the causative agents of malaria and are responsible for nearly a half million deaths each year.¹ In recent years, the parasite's mitochondrion has gained attention as a validated drug development target primarily for its molecular and functional divergence from the human mitochondrion and its involvement in ATP and nucleotide biosynthesis.^{2–4} This is exemplified by the successful development and clinical use of the cytochrome *b* inhibitor, atovaquone,⁵ as well as the promise of the dihydroorotate dehydrogenases (DHODH) inhibitor, DSM265,⁶ currently in clinical trials⁷ and another cytochrome *b* inhibitor, ELQ-300,⁸ entering preclinical trials.⁷

In contrast to the structurally and biochemically well-characterized mammalian mitochondrion, many aspects of the *Plasmodium* mitochondrion, such as function, bioenergetics, and associated metabolomics, remain unclear. Early work has shown that the malaria parasite utilizes glycolysis (catabolizing glucose to lactate) as its primary ATP source during erythrocytic development;^{9,10} however, comparative genomics studies have identified loci encoding enzymes of the electron transport chain (ETC) of the mitochondrion in the *Plasmodium falciparum* genome.^{11,12} In the *Plasmodium* ETC, at least five dehydrogenases have been described: type II NADH:oxidoreductase (NDH2), DHODH, malate:quinone oxidoreductase (MQO), glycerol-3-phosphate dehydrogenase (G3PDH), and succinate dehydrogenase (SDH of complex II) (Figure 1). These enzymes are assumed to supply electrons to downstream complex III, although to what extent each enzyme contributes as an electron donor is not fully understood.^{4,12} The proton gradient across the inner membrane, which complex V utilizes to drive ATP synthesis, has been observed in the *Plasmodium* mitochondrion,^{13–15} and complex V appears to be essential as disruption of genes encoding its subunits was unsuccessful.¹⁶ On the other hand, in the asexual blood stages, the *Plasmodium* mitochondrion is assumed to operate at a minimum level of oxidative phosphorylation (OXPHOS),¹⁷ and this is supported by a genomic study revealing the absence of some key subunits of complex V that are required for fully functioning ATP synthase activity.¹⁶ It has been proposed that the main function of the ETC in the blood stage parasites is to regenerate ubiquinone at complex III for DHODH, a key enzyme in the de novo pyrimidine biosynthesis pathway.^{18–20}

Oxygen consumption rate (OCR) at complex IV is an indicator of electron flow of the ETC and therefore has been used to bioenergetically assess the ETC enzymes and to characterize mitochondrial dysfunction and disease states. Compared to mammalian cells and other model organisms, the number of bioenergetic studies of malaria parasites are limited with only a few published reports discussing oxygen consumption using whole parasites^{14,15,19,21,22} or isolated mitochondria.^{23,24} Presumably, the main obstacle in conducting these experiments in malaria parasites is obtaining samples in sufficient quantity and quality to measure oxygen concentration by a Clark-type oxygen electrode. To overcome this challenge, we used an extracellular flux analyzer (XFe24 analyzer, Seahorse Bioscience, Billerica, MA, USA), which is capable of real-time analyses of mitochondrial respiration (i.e., oxygen consumption) and glycolysis in living cells and has been increasingly applied to a broad range of physiological and pathological research in systems of mammalian and other model species.²⁵ Similar to mitochondrial respiration, which alters oxygen concentration, cytosolic glycolysis releases organic acids outside of cells, causing acidification of the extracellular environment. With solid-state fluorescence sensors, the XFe24 analyzer simultaneously measures changes in oxygen concentration and pH with high sensitivity with a readout of OCR and extracellular acidification rate (ECAR), respectively. Furthermore, assay plates of the XFe24 analyzer are in a 24-well format, and each well has four drug delivery ports to inject effector molecules on a preset schedule. These features facilitate the analysis of mitochondrial responses to multiple effectors with improved throughput and high accuracy.

In this study, we developed a novel protocol utilizing the XFe24 analyzer to conduct bioenergetic studies of *P. falciparum* parasites isolated from RBCs. Using this protocol, we successfully monitored the kinetics of various mitochondrial substrates and metabolites (glucose, glutamate, G3P, succinate, malate, DHO, pyruvate, and ADP). To our knowledge, this is the first report to compare levels of electron flow from upstream mitochondrial enzymes in the ETC in malaria parasites by directly measuring OCR. We also tested various inhibitors targeting complex III, DHODH, and complex V to determine how these small molecules affect OCR. Lastly, we demonstrated the potential of this protocol as a novel methodology for target identification and mode of action (MOA) studies for mitochondrial inhibitors.

RESULTS AND DISCUSSION

Robust OCR and ECAR Fluctuations Portrait Cellular Respiration and Glycolysis Activities in Saponin-Freed Schizonts

Our attempt to measure extracellular fluxes using MACS-purified infected red blood cells (iRBCs) resulted in the observation of robust ECAR but little OCR with levels similar to those of uninfected RBCs (uRBCs) (Figure 2a). The failure of an OCR measurement could be due to a buffering effect of hemoglobin within RBCs. A low concentration of saponin (0.01%) has been used to lyse RBCs and obtain freed parasites seemingly intact.^{14,15,21} Using the saponin-freed parasites in the schizont stage, we found evidence for both ECAR and OCR (Figure 2b and c). To confirm that these observed OCR and ECAR truly represent activities of mitochondrial respiration and glycolysis, we treated parasites with 2-deoxy-D-

glucose (2-DG) and antimycin A and monitored the resultant OCR and ECAR fluctuations. 2-DG is known to competitively inhibit the reaction from glucose to glucose 6-phosphate in the glycolytic pathway. As expected, injection of 2-DG caused a sharp drop of the ECAR value, indicating that the observed ECAR represents the glycolytic activity occurring in parasites (Figure 2b). Antimycin A is a natural toxin produced by *Streptomyces bacteria* and is often used in bioenergetics research as a validated specific inhibitor of complex III. It binds to the ubiquinone reduction site (Qi site) of cytochrome *b* and interrupts electron transfer from complex III to cytochrome C (CytC) (Figure 1). Injection of antimycin A decreased OCR instantly but no significant change was observed for ECAR (Figure 2c). It is also known that reduction of oxygen consumption by antimycin A can be restored by chemically reducing CytC, and indeed, we observed an increase in OCR after injection of a reducing reagent: a mixture of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and ascorbate (Figure 2b). On the basis of these observations, we concluded that the OCR and ECAR fluctuations detected by the XFe24 analyzer represent mitochondrial respiration and glycolysis occurring in malaria parasites.

Next, we determined the optimal seeding density of parasites in a 24-well assay plate. Figure 2d shows that OCR and ECAR values increased proportionally to the parasite densities in a range from 5- to 13.4-million cells/well and either reached a plateau of maximal activity (OCR) or started declining (ECAR) with higher cell densities. At the highest densities (>13.4-million cells/well), parasites accumulated in multiple layers in the assay well and likely caused inaccurate measurements of oxygen and H⁺ concentrations. Thus, we determined the optimal seeding density for the schizont parasites to be 10–12-million cells/well, and all further assays we describe below were conducted with this range of cell density.

Mitochondria Assay Solution (MAS) Enables Isolated Studies of the ETC Dehydrogenases in Whole Cells

Armed with a successful assay to directly measure OCR and ECAR using whole parasites in unbuffered RPMI, we next sought to find suitable medium to assess activities of the ETC complexes separately. Because RPMI contains glucose (11 mM) and glutamine (2 mM), multiple ETC enzymes are likely activated, and the abundant electron supply they provide might mask fluctuation of an electron flow from an enzyme in question. We therefore tested a less complex assay buffer containing no fuel molecules. Figure 3a shows the OCR of saponin-freed schizonts seeded in three different media: MAS, MAS supplemented with glucose (15 mM), and MAS supplemented with succinate (10 mM). OCR values in MAS declined continuously over time and became lower than our observed nonrespiratory OCR level, suggesting that the parasites consumed endogenous fuel stocks and became metabolically inactive. On the other hand, addition of either glucose or succinate to the MAS resulted in stable and robust OCR similar to those observed in RPMI. As succinate is a substrate of SDH (complex II), this result also indicated that we were able to directly and selectively activate SDH and monitor its activity as an OCR fluctuation. Additionally, because both OCR and ECAR decreased continuously in MAS without any fuel molecules, we conducted a time-lapse analysis to determine preferable assay duration (Figure 3b). When glucose (2 mM) was added within 100 min after an assay started, robust elevations of

OCR and ECAR were observed; however, the same amount of glucose was not able to increase both OCR and ECAR after 130 min.

The Most Robust OCR and ECAR are Observed with Parasites at Schizont Stage during the Erythrocytic Cycle

We then further investigated if our assay protocol could be applied to parasites at earlier stages. Because the vast majority of protein and DNA synthesis occurs during the trophozoite and schizont stages of the erythrocytic cycle, the bioenergetic profile was expected to change with parasite development. Using highly synchronized cultures, we compared the OCR and ECAR increases of rings and trophozoites to those of schizonts by adding glucose (2 mM) to parasites in MAS. As expected, we found that the largest OCR and ECAR increases were observed with schizonts, indicating their high mitochondrial respiratory and glycolytic activities, whereas rings only showed a negligible level of OCR and ECAR increases (Figure 3c). We assume that the observed low OCR and ECAR levels represent the metabolically less active nature of rings, although there is a possibility that rings are more fragile than other stages and were not able to survive for long periods outside of the RBCs. Interestingly, trophozoites showed a similar level of ECAR but noticeably lower OCR than schizonts, possibly demonstrating their catabolic state. On the basis of these results, we decided to use parasites at schizont stage for further investigation. In addition, these findings clearly underscore the significance of parasite staging in obtaining reproducible bioenergetic data when measuring malaria parasites.

Succinate, Malate, G3P, DHO, and Glutamate Increase OCR but Not Pyruvate

The observation of OCR increase upon addition of succinate prompted us to examine whether this is also the case for substrates of other ETC dehydrogenases. Using the assay conditions developed above, 10 mM of malate, DHO, or G3P, which are substrates of MQO, DHODH, and G3PDH, respectively, was applied to freed schizonts in MAS, and consequent OCR changes were monitored. All substrates were found to initiate a robust and almost immediate increase of OCR. We next investigated whether glutamate and pyruvate have any influence on OCR. In the mitochondria, glutamate is converted to α -ketoglutarate,^{26–30} one of the components of the tricarboxylic acid cycle (TCA cycle), generating reducing equivalents to provide electrons to the ETC. In aerobic cells, pyruvate is a source of acetyl-CoA, an essential carbon source of the TCA cycle. As expected, the addition of glutamate resulted in a robust OCR increase similar to the tested ETC substrates. Interestingly, no OCR increase was observed after the addition of pyruvate (tested range: 5–40 mM as final), whereas malate (2.5–20 mM) increased OCR with each stepwise addition in the same experiment (Figure 4a).

We then titrated the tested metabolites to identify their optimal concentrations that achieve the highest OCR elevation by their target dehydrogenases. For example, succinate at six different concentrations (0, 5, 10, 20, 40, and 60 mM) was added to parasites in MAS, and the resultant OCR increases were calculated. As shown in Figure 4b, the degree of OCR induction reached a maximum level and plateaued at 40 mM, which was determined as the optimal concentration for succinate. All other compounds exhibited similar trends (Figure 4c–f), and each optimal concentration was determined. Next, for comparing the highest

achievable OCR among the tested metabolites, parasites were treated with one of these substrates at their corresponding optimized concentration. As a result, we found that G3P gave the highest overall OCR and did so at a lower concentration (8 mM) relative to that of the other substrates (Figure 4g).

Malate Increases OCR with or without Digitonin but Glucose Does Not Increase OCR in a Fully Permeabilized Condition

The tested mitochondrial substrates possess polar functional groups, such as carboxyl and phosphate groups, which makes their free passage through the cytoplasmic membrane unlikely. Because we added 2 μ M of digitonin to our assay media following the previous reports of OCR measurement in *P. yoelii*¹⁵ and *P. berghei*,¹⁴ it was assumed that 2 μ M of digitonin permeabilized the membrane, allowing entry of polar substrates into the cell. However, the observation of simultaneous OCR and ECAR readings indicates that the cytosolic enzymes required for glycolysis remain in the cell and that the cytoplasmic membrane was not fully permeabilized. To study this seemingly contradictory observation in detail, we examined the effects of digitonin permeabilization on both OCR and ECAR. Figure 5a shows OCR and ECAR values of parasites treated with various concentration of digitonin in MAS supplemented with glucose (6 mM). With digitonin at 300 μ M and higher, the ECAR values were considerably reduced, indicating that the cytoplasmic membrane was fully permeabilized and that the glycolytic enzymes were released from the cells. This result is consistent with previous work shown by Western blotting that a cytosolic protein was released by digitonin at 170 μ M and higher concentrations in *P. falciparum*.³¹ As expected, the OCR values also decreased in a similar manner, and with digitonin at 300 μ M and higher, the OCR reached the same level as when the ETC electron flow was stopped by the addition of antimycin A. This result also suggested that glycolytic catabolites entered the mitochondrion and activated components in the TCA and/or the ETC.

Next, we compared OCR inductions by glucose and malate in parasites with and without digitonin permeabilization. As shown in Figure 5b, robust malate-induced OCR was observed regardless of the digitonin concentration. In contrast, glucose did not increase OCR in parasites fully permeabilized with digitonin (300 μ M). This result clearly shows that malate crosses the cytoplasmic membrane to activate MQO in the mitochondrion without digitonin permeabilization and that it does not require cytosolic components like glucose. Additionally, this experiment demonstrates that mitochondrial activity remains intact with the higher concentration of digitonin and that the lack of glucose-derived OCR is not merely a consequence of digitonin disrupting complex formation in the ETC. It is also noteworthy that pyruvate failed to increase OCR not only under the regular assay condition containing 2 μ M digitonin (Figure 4a) but also under the fully permeabilized condition with 300 μ M digitonin (Figure 5b).

Glucose Regulates a Metabolic Shift between Mitochondrial Respiration and Glycolysis

In addition to mitochondrial substrates, we conducted titrations for glucose and observed different effects on ECAR and OCR. Figure 6a shows OCR and ECAR values approximately 30 min after addition of glucose and reveals that ECAR increased proportionally with increasing doses of glucose in the tested range. The kinetic ECAR graph

(Figure 6b) illustrates that 2 and 4 mM doses initially led to elevated ECAR levels similar to the 6 mM dose but resulted in depletion of glucose during the assay period, revealing that the 6 mM dose was required to sustain the highest level of glycolysis. On the other hand, the maximum level of OCR was achieved with lower doses of glucose (0.5–2 mM) and higher doses resulted in lower OCR (Figure 6a). The kinetic OCR graph (Figure 6b) shows that all concentrations initially elevated OCR to similar levels, but the OCR induced with 4 and 6 mM glucose declined within ~10 min. The OCR induced with 2 mM glucose showed the same decreasing trend as observed with the 4 and 6 mM doses but then increased again during the experimental period. In contrast, parasites treated with 0.5 and 1 mM maintained the elevated OCR levels. The observation that OCR and ECAR had different responses to glucose levels suggests that glucose functions as a switch between mitochondrial respiration and glycolysis. Thus, in environments with abundant glucose, such as culture medium or the bloodstream, parasites depend on glycolysis for energy production. However, once glucose levels become lower, e.g., at 1 mM, parasites can compensate for the shortage of energy by increasing oxygen consumption.

***Plasmodium* Complex V is Coupled with the ETC with a High Level of Proton Leak**

As previously mentioned, the presence and the role of OXPHOS in *Plasmodium* have been debated.^{3,12,17,32} To address this unresolved question, we sought to directly measure OXPHOS by monitoring OCR fluctuations upon addition of ADP. ADP at seven different concentrations (0, 0.75, 1.5, 3, 6, 12, and 24 mM) was given to parasites seeded in unbuffered RPMI. With ADP at 1.5 mM and higher, robust OCR increases were observed, illustrating that complex V is coupled with the ETC, i.e., the presence of OXPHOS (Figure 7a). This ADP-induced OCR increase was also observed in MAS supplemented with glucose (11 mM). In our experiment, a stable 50–75% increase over the basal level of OCR was observed with 6–24 mM ADP. By comparison, in a similar assay using mouse liver mitochondria, 1–4 mM ADP induced a stable 5-fold elevation of OCR.³³ The observed low level of OCR increase presumably reflects the poor efficiency of *Plasmodium* OXPHOS in the asexual blood stage, although there is a possibility that low permeability of ADP under the current assay conditions might necessitate the apparently excessive amount of ADP.

Oligomycin A is a well-known complex V inhibitor and is often used in bioenergetic studies to determine the proportion of OCR devoted to mitochondrial ATP synthesis and the remaining proton leak. However, it was reported that oligomycin A is inactive against *Plasmodium* complex V³⁴ and that *P. falciparum* has an incomplete Fo site,¹⁶ which oligomycin A is known to bind to in mammalian complex V. Therefore, we first examined if oligomycin A is capable of decreasing OCR and what concentration is required. We found that 10 μ M of oligomycin A decreased OCR in unbuffered RPMI and that the inhibition was more prominent and dose-responsive in the presence of ADP (Figure 7b and d).

Next, we characterized the OXPHOS in malaria parasites using ADP, oligomycin A, FCCP, and antimycin A. FCCP, a validated uncoupling agent, dissipates the proton gradient across the inner membrane and is often used to quantify the mitochondrial reserve capacity (or maximum respiratory capacity) by uncoupling the oxygen consumption at complex IV from the functionality of complex V. Antimycin A stops the electron flow of the ETC at complex

III and reveals oligomycin A-insensitive OCR, i.e., the level of proton leak. Figure 7c shows the OCR fluctuation by sequential addition of these reagents to parasites in unbuffered RPMI. As expected from the previous experiments, ADP (24 mM) addition resulted in a small but robust OCR increase, which was diminished by oligomycin A (10 μ M), suggesting this OCR elevation was caused by OXPHOS. The subsequent addition of FCCP (2 μ M) elevated OCR again, but the induction level was small and similar to that observed by the addition of ADP. Finally, addition of antimycin A (1 μ M) resulted in dramatically decreased OCR levels, indicating a high level of proton leak. Additionally, for comparing OCR levels without the influence of sample fatigue over the experimental period, parasites were treated with these reagents at the same time, showing similar results (Figure 7d). Thus, in unbuffered RPMI, *Plasmodium* mitochondria exhibited OXPHOS activity with a limited reserve capacity and high proton leak.

OCR Readout can be Used for Target and MOA Study of Mitochondrial Inhibitors

Because OCR fluctuation reflects the activity of the ETC enzymes, we assumed that it would also reveal how mitochondrial inhibitors affect the ETC (i.e., at which complex/point in the chain) and therefore reveal their MOA. First, we investigated the effect of cytochrome *b* inhibitors on OCR levels. Antimycin A^{3,35} and IDI-5918³⁶ inhibit cytochrome *b* by binding to its Qi site, and atovaquone,³⁷ decoquinatone,³⁸ and BTZ-1³⁹ inhibit it by binding to the ubiquinol oxidation site (Qo site). These inhibitors were added in three doses to parasites in unbuffered RPMI, and the resultant OCR changes were monitored for 45 min. We found that all compounds decreased OCR in a dose-dependent manner with differing kinetics (Figure 8a–e). Atovaquone and antimycin A were able to decrease OCR to the background level at 0.1 and 0.3 μ M, respectively, whereas other inhibitors required higher doses to decrease OCR despite their high potency (nanomolar to subnanomolar EC₅₀) in whole cell assays. Decoquinatone exhibited the slowest kinetics and did not achieve complete inhibition within the experimental duration.

The finding that OCR fluctuation represents inhibition of cytochrome *b*/complex III inspired us to use this assay as a tool to identify the targets of mitochondrial inhibitors. We observed that the DHODH inhibitor, genz-669178, failed to lower OCR in unbuffered RPMI, an assay condition in which electrons from other dehydrogenases likely mask the effect of genz-669178 on the electron flow from DHODH (Figure 9a). However, OCR reduction by genz-669178 should be observable if DHODH is the only source of electrons for the ETC. To test this hypothesis, we treated parasite samples individually with one of the four substrates of the ETC enzymes (G3P, succinate, malate, or DHO) at their predetermined optimal concentrations and then added genz-669178 or antimycin A. As expected, genz-669178 only decreased the OCR that was induced by DHO, whereas antimycin A lowered the OCR in all conditions (Figure 9b). This experiment clearly indicates that genz-669178 selectively inhibits DHODH but not the other ETC enzymes. It also demonstrates the pan-inhibitory activity of antimycin A due to its inhibition of complex III, a chokepoint for electrons from all dehydrogenases on the ETC.

Lastly, using *Sc*DHODH-expressing parasites, we measured OCR responses to atovaquone, antimycin A, and BTZ-1. This transgenic strain expresses ubiquinone-independent

Saccharomyces cerevisiae DHODH in cytoplasm and is known to be resistant to cytochrome *b* inhibitors.²⁰ As shown in Figure 9c, OCR reduction was observed in the same way as Dd2, demonstrating that the electron flow of the ETC was completely abolished by these inhibitors despite their lack of activity in the growth assay ($EC_{50} > 20 \mu M$). This result not only emphasizes that what we observed by OCR fluctuation is distinct from growth inhibition but also suggests that the mode of action for the cytochrome *b* inhibitors is mainly the disruption of P/DHODH resulting from depletion of ubiquinone, which is regenerated at complex III.

Conclusions and Implications

Seahorse extracellular flux analyzers have been increasingly utilized to measure the metabolic profiles of mammalian cells and model organisms including *Drosophila*⁴⁰ and parasitic worms,^{41,42} but to our knowledge, this report is the first application on malaria parasites. Our assay protocol using the XFe24 analyzer enabled us to monitor activities of both glycolysis and mitochondrial respiration in *P. falciparum* simultaneously and to analyze the impacts of various metabolites and inhibitors in real time. Its capability of accommodating several assay conditions in a single experiment was advantageous for comparative studies.

Using the optimized assay platform, the effects of mitochondrial substrates and metabolites were quantified, and valuable information for understanding *Plasmodium* bioenergetics and metabolomics was obtained. For example, comparative analyses of maximum achievable OCR using the corresponding substrates at the optimized concentrations revealed that G3PDH had the largest potential as an electron donor among tested mitochondrial dehydrogenases despite having been previously considered a minor contributor³⁹ (Figure 4g). Also, our observation of the robust glutamate-induced OCR elevation demonstrated a clear association of the TCA cycle and the ETC (Figure 4d). Furthermore, the discovery of oligomycin A-sensitive OCR elevation by ADP provided direct experimental evidence for the existence of OXPHOS (Figure 7). However, quantification of the high proton leak and the low reserve capacity revealed its inefficient nature at the schizont stage. In addition, oligomycin A decreased only minimum OCR in RPMI without ADP (24 mM) supplementation. These findings indicate that malaria parasites at the schizont stage conduct little OXPHOS at the basal state, supporting the common assumption that asexual blood stage parasites mainly depend on glycolysis for energy generation.

The observation that pyruvate failed to increase OCR (Figure 4a and 5b), in contrast to other tested substrates (Figure 4b–f), indicates that pyruvate is not incorporated into the TCA cycle. In *Plasmodium* spp., incorporation of pyruvate-derived acetyl-CoA into the TCA cycle or the source of acetyl-CoA has been disputed.^{3,12,17,43} In aerobic cells, pyruvate dehydrogenase (PDH) converts glycolytic pyruvate to acetyl-CoA. However, this scenario has been considered unlikely for malaria parasites, which express PDH in the apicoplast but not in the mitochondrion.⁴⁴ Likewise, the incorporation of fatty acids and branched-chain amino acids, which are also a source of acetyl-CoA in other organisms, has been controversial because of the absence of the enzymes needed for those catabolic reactions.²⁷ On the other hand, several comparative metabolomics studies using [U-¹³C]glucose have

demonstrated the entry of glycolytic products into the TCA cycle via acetyl CoA as evidenced by observations of 2 atomic mass unit-shifted acetyl CoA and citrate.^{26,28–30} Recently, it was reported that a branched-chain keto acid dehydrogenase (BCKDH) possesses PDH-like function²⁸ and converts pyruvate to acetyl-CoA in *T. gondii* and *P. berghei*.²⁷ We demonstrated that pyruvate does not stimulate the OCR to the same extent as G3P, malate, succinate, DHO, and glutamate. These results are in contrast to the reports using ¹³C tracer experiment in *T. gondii* and *P. berghei*.²⁷ This could indicate a biological difference in *P. falciparum* or could be due to the differential enzyme activity or permeability of pyruvate in our assay. Further experimentation will be required to resolve this issue. [U-¹³C]glucose tracer experiments revealed that glucose is also catabolized to G3P and malate in the cytoplasm via dihydroxyacetone phosphate and phosphoenolpyruvate, respectively.³⁰ As pyruvate does not increase OCR in our assay, G3P and malate might be the actual agents responsible for the observed “glucose-induced” OCR (Figure 6). This hypothesis is consistent with the finding that glucose no longer stimulates OCR in fully permeabilized cells where cellular components necessary for glycolysis are lost (Figure 5).

One of the notable advantages of our protocol is the capability of simultaneously measuring OCR and ECAR. It revealed that diminution of the glucose supply caused a metabolic shift from glycolysis to OXPHOS (Figure 6), a similar phenomenon as seen with *S. cerevisiae*.⁴⁵ This finding indicates that parasites are metabolically flexible and have the ability to respond to environmental cues. Proteome and transcriptome data sets have revealed the upregulation of several enzymes involved in the TCA cycle and OXPHOS in gametocytes and ookinetes,⁴⁶ sporozoites,⁴⁷ and liver stage parasites.⁴⁸ The increased TCA catabolism in gametocytes was also observed in a metabolomics study.²⁹ Furthermore, genetic approaches have been used to demonstrate that SDH, complex V, and several enzymes in the TCA cycle are dispensable in the asexual blood stage but essential in the mosquito stage.^{49,50} These studies indicate that parasites undergo dramatic metabolic shifts between glycolysis and mitochondrial respiration during stage transitions. Nutrient sensing might help parasites adapt to drastic environmental changes by regulating this metabolic shift.

In addition to metabolomics studies, the extracellular flux analysis provides a novel methodology to characterize mitochondrial inhibitors. As described in Figure 8, cytochrome *b* inhibitors reduced OCR in a dose-dependent manner. The inhibitory concentrations in the cellular growth assays and the extracellular flux assays are different, and this may be explained by the difference in the two assays. The extracellular flux measurements occur in a 45 min assay, whereas the growth inhibition assays are determined over a 72 h time frame. Binding kinetics, cell permeability, and enzyme kinetics will influence the inhibitory concentrations differentially in the two assays. The important observation is the dose dependency.

Identifying the target of small molecule inhibitors of parasite growth is an important activity with regard to both potential drug discovery and development. This assay gives us a new and innovative tool to find potential inhibitors of mitochondrial respiration among those small molecules that inhibit parasite growth. This has the potential for use as a secondary screen to identify molecules specific for DHODH, complex III, SDH, MQO, G3PDH, and NDH2. In this publication, we have demonstrated the proof of this principle by testing known

inhibitors of DHODH and cytochrome *b* (complex III) and demonstrating that this bioenergetic probe can precisely identify the step in the ETC inhibited by a small molecule (Figure 9b). This is a major advantage over the currently used method of bypassing mitochondrial activity entirely through cytoplasmic expression of yeast DHODH.^{20,36} Furthermore, the OCR analysis would serve as a valuable tool for MOA study of mitochondrial antimalarials. For example, we demonstrated that genz-669178 did not lower OCR (or ECAR) in unbuffered RPMI (Figure 9a) and concluded that its antimalarial activity is not mediated through disruption of the ETC but is due to a direct effect on de novo pyrimidine biosynthesis. Even more noteworthy, we showed that the MOA of cytochrome *b* inhibitors is also due to the disruption of pyrimidine biosynthesis. These compounds perturb cytochrome *b* (complex III) to regenerate ubiquinone, which is essential for the activities of the ETC dehydrogenases. Therefore, their MOA could be a direct inhibition of cytochrome *b* as well as the resultant disruptions of the ETC dehydrogenases. Treatment with cytochrome *b* inhibitors reduced OCR in the *ScDHODH* line similarly to that of the *Dd2* line (Figure 9c) despite the fact that the compounds are inactive against the *ScDHODH* line in the growth assay. This result clearly indicates that *ScDHODH*, which utilizes fumarate instead of ubiquinol, plays a significant role in overcoming the disruptions in OCR. With this finding, we conclude that the MOA of cytochrome *b* inhibitors is pyrimidine biosynthesis inhibition caused by depletion of ubiquinone.

In prior studies of the ETC dehydrogenases and the OXPHOS, several groups have examined the impact of mitochondrial substrates and inhibitors on oxygen consumption in *Plasmodium* spp. Utilizing Clark-type oxygen electrodes, these previously published data are mostly consistent with our observations and conclusions. There are differences in the parasite species tested and in the experimental design when compared to the work reported here, and we assume that this explains differences in specific observations. For example, using isolated *P. falciparum* mitochondria, Takashima et al.²³ observed DHO-dependent oxygen consumption but did not see succinate dependency. Consistent with our results, Uyemura et al.^{14,15} observed that oxygen consumption increased in the presence of ADP in rodent malaria models and showed the presence of OXPHOS, although at a lower concentration than for the *P. falciparum* system we observed. Krungkrai et al.^{19,32} demonstrated that both antimycin A and atovaquone inhibited oxygen consumption in *P. falciparum*, albeit at somewhat different inhibitory concentrations than we observed.

Currently, selective inhibitors are only available for DHODH and cytochrome *b*, and we were not able to confirm that our assay platform is applicable to inhibitors that target other ETC components. We look forward to the possibility of testing this as novel potential inhibitors are identified. With these inhibitors in hand, our next challenge would be to establish a method to achieve truly selective activation of target enzymes. For example, activation of SDH with succinate would drive the TCA cycle and cause subsequent activation of MQO and possibly NDH2. For preventing this unwanted activation, selective inhibitors of MQO and NDH2 need to be added. Nevertheless, our assay protocol utilizing whole parasites, and an Xfe24 analyzer offers the opportunity to conduct various biochemical assays in a physiologically relevant microenvironment and provide valuable novel methodology for *Plasmodium* metabolomics and MOA study of antimalarial agents.

METHODS

Parasite Culture

The erythrocytic stages of *P. falciparum* (Dd2 and Dd2-scDHODH) were cultured by standard methods in RPMI 1640 medium (Life Technologies) supplemented with 5% human O+ erythrocytes, 28 mM NaHCO₃, 25 mM HEPES, 0.5% (w/v) AlbuMAX II (Life Technologies), 50 mg/mL of hypoxanthine, and 25 µg/mL of gentamycin. Human blood was supplied from Research Blood Components or Interstate Blood Bank. Cultures were grown in a gas mixture of 5% O₂, 5% CO₂, and 90% N₂ and regularly synchronized by 5% sorbitol treatment.⁵¹ Highly synchronized culture for bioenergetics profiling of developmental stages was obtained by a combination of sorbitol synchronization and heparin treatment. After sorbitol synchronization, the obtained ring stage parasites were cultured with the presence of heparin sodium salt from porcine intestinal mucosa (230 µg/mL; Sigma-Aldrich) until the majority of parasites developed to the late schizont stage. Then, heparin was removed from culture for 6 h, allowing merozoites to invade erythrocytes. The culture was kept with heparin for another 18 h and washed.

Assay Media

Two assay media were employed for the analyses: RPMI 1640 medium (Gibco 31800–089) without any supplementation (unbuffered RPMI) or mitochondria assay solution (MAS). MAS was prepared as a 3× solution and composed of mannitol (660 mM), sucrose (210 mM), KH₂PO₄ (30 mM), MgCl₂ (15 mM), HEPES (6 mM), EGTA (3 mM), and fatty acid-free BSA (0.6% w/v). MAS has been used when looking at bioenergetics of isolated mitochondria and permeabilized mammalian cells. It was designed to provide the right osmolarity for mitochondrial studies. The manufacturer recommends the use of MAS containing a small amount of HEPES as it helps to stabilize ECAR values and to obtain more precise rates. Both media were adjusted to pH 7.4 and stored at 4 °C.

Reagents

ADP monopotassium salt dehydrate, ascorbic acid (ascorbate), L-dihydroorotic acid (DHO), sn-glycerol 3-phosphate bis(cyclohexylammonium) salt (G3P), glucose, L-glutamic acid potassium salt monohydrate (glutamate), malic acid (malate), pyruvic acid (pyruvate), succinic acid (succinate), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), 2-deoxy-D-glucose (2-DG), antimycin A, atovaquone, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP), and decoquinate were purchased from Sigma-Aldrich (St. Louis, MO). Oligomycin A was purchased from Calbiochem (San Diego, CA). BZT-1, genz-669178, and IDI-5918 were provided by Prof. Ralph Mazitschek (Massachusetts General Hospital, MA), Genzyme (Waltham, MA), and the Broad Institute (Cambridge, MA), respectively.

Stock Solution of Test Compounds

The following stock solutions were prepared at the indicated concentrations in deionized water or unbuffered RPMI, adjusted to pH 7.4 with KOH, filtered, and stored at –20 °C in aliquots: DHO (2 M in H₂O), glucose (1 M in H₂O), glutamate (2 M in H₂O), G3P (0.2 M in

H₂O), malate (2 M in H₂O), pyruvate (1 M in H₂O), succinate (1 M in H₂O), 2-DG (0.5 M in RPMI), ADP (0.3 M in RPMI), and a mixture of TMPD and ascorbate (3 and 5 mM in RPMI, respectively). Stock solutions of antimalarial compounds and FCCP were prepared in DMSO at 1 mM and stored at -20 °C in aliquots.

General Procedure of Extracellular Flux Analysis Using XFe24 Analyzer

All assays were conducted according to the manufacturer's manual with some modifications. A sensor cartridge was hydrated overnight or longer in XF Calibrant Solution at 37 °C. On the day of the assay, digitonin (2 µM) was added freshly to medium, and the medium was mixed and filtered. Injection solutions containing test compounds were prepared in assay medium at 10× of the final concentration by combining the aqueous stock solution, H₂O, and 3× MAS or adding DMSO stock solution to assay media (1× MAS or unbuffered RPMI). The prepared 10× solution was filtered before use. The stock solution of 2-DG and a mixture of TMPD and ascorbate were used at 10×. After exchanging the XF Calibrant Solution to a new batch, the 10× injection solutions (50, 56, 62, and 68 µL for the first, second, third, and fourth injections, respectively) were loaded in the reagent delivery chambers of the sensor. While calibrating the sensor cartridge, parasites were freed from RBCs and placed in an assay plate in the following way: Typically, packed, infected RBCs (4 mL) at 4% parasitemia (schizonts) were mixed with 0.01% saponin in PBS (200 mL) for 1 min and centrifuged at 800 rcf for 3 min. For the assays using parasites at early stages, saponin-lysed, infected RBCs were centrifuged again at 3000 rcf for trophozoites or at 6000 rcf for rings. The obtained parasite pellets were washed with assay medium twice and resuspended in assay medium. After counting cells using a hemocytometer, the cell density was adjusted to 10 to 12-million cells/100 µL. The parasites in assay medium were then transferred into an XF24-well microplate at 100 µL/well with wells pretreated with CellTak cell and tissue adhesive (Fisher Scientific, CB-40241) according to the manufacturer's manual. After centrifugation at 52 rcf for 5 min with slow acceleration and no braking, 350 µL of assay medium was slowly added to all wells, and the assay plate was loaded into the flux analyzer to start measurements (mix time: 30 s; wait time: 90 s; measure time: 3 min). In an assay plate, 2–4 wells were used for background correction.

Normalization Using DNA Content

For OCR and ECAR values to be normalized to parasite DNA content, SYBR Green I fluorescent dye (Invitrogen S7563) was used. The parasite suspension used in the assay and standard Lambda DNA (Invitrogen) was serially six-point diluted at 1:1 using PBS. SYBR green dye (10×) in lysis buffer [0.16% (w/v) saponin, 20 mM Tris-HCl, 5 mM EDTA, 1.6% (v/v) Triton X-100, pH 7.4] was dispensed at 10 µL/well in a 384-well black clear bottom plate, to which serial dilutions of standard Lambda DNA or parasite suspension were added at 40 µL/well in duplicate. After overnight incubation at room temperature, fluorescence of each samples was read (excitation and emission wavelengths of 485 and 535 nm, respectively), and parasite DNA was calculated using the standard curve obtained by Lambda DNA.

Statistical Analysis

All statistical analysis was conducted using ordinary one-way ANOVA with Holm–Sidak’s multiple comparisons test using Prism Software version 6 (GraphPad).

In Vitro Drug Sensitivity and EC₅₀ Determination

Drug susceptibility was measured by growth assay as previously reported.³⁶ Briefly, synchronized ring stage parasites were cultured in the presence of 12-point serial dilutions of the test compounds in triplicate in 384-well black clear-bottom plates for 72 h. SYBR Green I fluorescent dye (Invitrogen S7563) was added, and after overnight incubation at room temperature, fluorescence was read (excitation and emission wavelengths of 485 and 535 nm, respectively). EC₅₀ values were calculated using a nonlinear regression curve fit in Prism Software version 6 (GraphPad). The assay was replicated 3–5 times.

Acknowledgments

We thank Prof. R. Mazitschek (Massachusetts General Hospital, MA), Genzyme (Waltham, MA), and the Broad Institute (Cambridge, MA) for BZT-1, genz-669178, and IDI-5918, respectively. We are also thankful to Prof. B. A. Burleigh (Harvard T.H. Chan School of Public Health) and Dr. K. Caradonna (Seahorse Bioscience) for advice on assay development and thoughtful comments on the manuscript, and Dr. A. K. Lukens, Dr. P. A. Magistrado and R. Mandt (Harvard T.H. Chan School of Public Health) and Dr. N. Kato (The Broad Institute) for helpful discussions and assistance with the manuscript. This work was supported by National Institute of Health grant R01 AI093716 and Harvard Malaria Initiative support from the ExxonMobil Foundation.

ABBREVIATIONS

BCKDH	branched-chain keto acid dehydrogenase
2-DG	2-deoxy-D-glucose
DHO	dihydroorotate
DHODH	dihydroorotate dehydrogenase
ECAR	extracellular acidification rate
ETC	electron transport chain
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
G3P	glycerol 3-phosphate
G3PDH	glycerol-3-phosphate dehydrogenase
MAS	mitochondria assay solution
MOA	mode of action
MQO	malate:quinone oxidoreductase
NDH2	NADH:ubiquinone oxidoreductase
OCR	oxygen consumption rate

OXPHOS	oxidative phosphorylation
PDH	pyruvate dehydrogenase
Qi	ubiquinone reduction
Qo site	ubiquinol oxidation site
RBCs	red blood cells
iRBCs	infected red blood cells
uRBCs	uninfected red blood cells
SDH	succinate:ubiquinone oxidoreductase
TCA cycle	tricarboxylic acid cycle
TMPD	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine dihydrochloride

References

1. World Health Organization. World Malaria Report. 2014. p. 1-142.
2. Vaidya AB. Mitochondrial and Plastid Functions as Antimalarial Drug Targets. *Curr. Drug Targets: Infect. Disord.* 2004; 4(1):11–23. [PubMed: 15032631]
3. Torrentino-Madamet M, Desplans J, Travaille C, Jammes Y, Parzy D. Microaerophilic respiratory metabolism of *Plasmodium falciparum* mitochondrion as a drug target. *Curr. Mol. Med.* 2010; 10(1):29–46. [PubMed: 20205678]
4. Nixon GL, Pidathala C, Shone AE, Antoine T, Fisher N, O'Neill PM, Ward SA, Biagini GA. Targeting the mitochondrial electron transport chain of *Plasmodium falciparum*: new strategies towards the development of improved antimalarials for the elimination era. *Future Med. Chem.* 2013; 5(13):1573–1591. [PubMed: 24024949]
5. Fry M, Pudney M. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthoquinone (566C80). *Biochem. Pharmacol.* 1992; 43(7):1545–1553. [PubMed: 1314606]
6. Coteron JM, Marco M, Esquivias J, Deng X, White KL, White J, Koltun M, Mazouni Elf, Kokkonda S, Katneni K, et al. Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors with clinical candidate potential. *J. Med. Chem.* 2011; 54(15):5540–5561. [PubMed: 21696174]
7. The GHIT Fund Advancing Portfolio | Global Health Innovative Technology Fund.
8. Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ, Shackleford DM, Saenz FE, et al. Quinolone-3-diarylethers: a new class of *antimalarial drug*. *Sci. Transl. Med.* 2013; 5(177):177ra37.
9. Bryant C, Voller A, Smith M. The Incorporation of Radioactivity from [¹⁴C] Glucose into the Soluble Metabolic Intermediates of Malaria Parasites. *Am. J. Trop. Med. Hyg.* 1964:515–519. [PubMed: 14196045]
10. Sherman, IW. Carbohydrate metabolism of asexual stages. In: Sherman, IW., editor. *Malaria: Parasite biology, pathogenesis and protection*. ASM; Washington, DC: 1998. p. 135-143.
11. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature.* 2002; 419(6906):498–511. [PubMed: 12368864]
12. van Dooren GG, Stimmler LM, McFadden GI. Metabolic maps and functions of the *Plasmodium mitochondrion*. *FEMS Microbiol. Rev.* 2006; 30(4):596–630. [PubMed: 16774588]

13. Srivastava IK, Rottenberg H, Vaidya AB. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *J. Biol. Chem.* 1997; 272(7): 3961–3966. [PubMed: 9020100]
14. Uyemura SA. Oxidative Phosphorylation, Ca²⁺ Transport, and Fatty Acid-induced Uncoupling in Malaria Parasites Mitochondria. *J. Biol. Chem.* 2000; 275(13):9709–9715. [PubMed: 10734123]
15. Uyemura SA, Luo S, Vieira M, Moreno SNJ, Docampo R. Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in *Plasmodium yoelii* yoelii mitochondria in situ. *J. Biol. Chem.* 2004; 279(1):385–393. [PubMed: 14561763]
16. Nina PB, Morrissey JM, Ganesan SM, Ke H, Pershing AM, Mather MW, Vaidya AB. ATP Synthase Complex of *Plasmodium falciparum*: DIMERIC ASSEMBLY IN MITOCHONDRIAL MEMBRANES AND RESISTANCE TO GENETIC DISRUPTION. *J. Biol. Chem.* 2011; 286(48):41312–41322. [PubMed: 21984828]
17. Vaidya AB, Mather MW. Mitochondrial Evolution and Functions in Malaria Parasites. *Annu. Rev. Microbiol.* 2009; 63(1):249–267. [PubMed: 19575561]
18. Gutteridge WE, Dave D, Richards WH. Conversion of dihydroorotate to orotate in parasitic protozoa. *Biochim. Biophys. Acta, Gen. Subj.* 1979; 582(3):390–401.
19. Krungkrai J, Burat D, Kudan S, Krungkrai S, Prapunwattana P. Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite. *Plasmodium falciparum*. *Southeast Asian. J. Trop. Med. Public Health.* 1999; 30(4):636–642.
20. Painter HJ, Morrissey JM, Mather MW, Vaidya AB. Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. *Nature.* 2007; 446(7131):88–91. [PubMed: 17330044]
21. Murphy AD, Doeller JE, Hearn B, Lang-Unnasch N. *Plasmodium falciparum*: cyanide-resistant oxygen consumption. *Exp. Parasitol.* 1997; 87(2):112–120. [PubMed: 9326886]
22. Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Hinrichs D, Riscoe MK. Antimalarial quinolones: synthesis, potency, and mechanistic studies. *Exp. Parasitol.* 2008; 118(4):487–497. [PubMed: 18082162]
23. Takashima E, Takamiya S, Takeo S, Mi-ichi F, Amino H, Kita K. Isolation of mitochondria from *Plasmodium falciparum* showing dihydroorotate dependent respiration. *Parasitol. Int.* 2001; 50(4): 273–278. [PubMed: 11719114]
24. Krungkrai J, Kanchanarithsak R, Krungkrai SR, Rochanakij S. Mitochondrial NADH Dehydrogenase from *Plasmodium falciparum* and *Plasmodium berghei*. *Exp. Parasitol.* 2002; 100(1):54–61. [PubMed: 11971654]
25. Salabei JK, Gibb AA, Hill BG. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. *Nat. Protoc.* 2014; 9(2):421–438. [PubMed: 24457333]
26. Ke H, Lewis IA, Morrissey JM, McLean KJ, Ganesan SM, Painter HJ, Mather MW, Jacobs-Lorena M, Llinás M, Vaidya AB. Genetic Investigation of Tricarboxylic Acid Metabolism during the *Plasmodium falciparum* Life Cycle. *Cell Rep.* 2015; 11(1):164–174. [PubMed: 25843709]
27. Oppenheim RD, Creek DJ, MacRae JJ, Modrzyńska KK, Pino P, Limenitakis J, Polonais V, Seeber F, Barrett MP, Billker O, et al. BCKDH: The Missing Link in Apicomplexan Mitochondrial Metabolism Is Required for Full Virulence of *Toxoplasma gondii* and *Plasmodium berghei*. *PLoS Pathog.* 2014; 10(7):e1004263. [PubMed: 25032958]
28. Cobbold SA, Vaughan AM, Lewis IA, Painter HJ, Camargo N, Perlman DH, Fishbaugher M, Healer J, Cowman AF, Kappe SHI, et al. Kinetic Flux Profiling Elucidates Two Independent Acetyl-CoA Biosynthetic Pathways in *Plasmodium falciparum*. *J. Biol. Chem.* 2013; 288(51): 36338–36350. [PubMed: 24163372]
29. MacRae JJ, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S, Bottova I, Tilley L, McConville MJ. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite *Plasmodium falciparum*. *BMC Biol.* 2013; 11(1):67. [PubMed: 23763941]
30. Storm J, Sethia S, Blackburn GJ, Chokkathukalam A, Watson DG, Breitling R, Coombs GH, Müller S. Phosphoenolpyruvate Carboxylase Identified as a Key Enzyme in Erythrocytic *Plasmodium falciparum* Carbon Metabolism. *PLoS Pathog.* 2014; 10(1):e1003876. [PubMed: 24453970]

31. Bulusu V, Jayaraman V, Balaram H. Metabolic Fate of Fumarate, a Side Product of the Purine Salvage Pathway in the Intraerythrocytic Stages of *Plasmodium falciparum*. *J. Biol. Chem.* 2011; 286(11):9236–9245. [PubMed: 21209090]
32. Krungkrai J. The multiple roles of the mitochondrion of the malarial parasite. *Parasitology.* 1999; 129(5):511–524.
33. Rogers GW, Brand MD, Petrosyan S, Ashok D, Elorza AA, Ferrick DA, Murphy AN. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS One.* 2011; 6(7):e21746. [PubMed: 21799747]
34. Mather MW, Morrissey JM, Vaidya AB. Hemozoin-free *Plasmodium falciparum* mitochondria for physiological and drug susceptibility studies. *Mol. Biochem. Parasitol.* 2010; 174(2):150–153. [PubMed: 20674615]
35. di Rago JP, Colson AM. Molecular basis for resistance to antimycin and diuron, Q-cycle inhibitors acting at the Qi site in the mitochondrial ubiquinol-cytochrome c reductase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 1988; 263(25):12564–12570. [PubMed: 2842335]
36. Lukens AK, Heidebrecht RW, Mulrooney C, Beaudoin JA, Comer E, Duvall JR, Fitzgerald ME, Masi D, Galinsky K, Scherer CA, et al. Diversity-oriented synthesis probe targets *Plasmodium falciparum* cytochrome b ubiquinone reduction site and synergizes with oxidation site inhibitors. *J. Infect. Dis.* 2015; 211(7):1097–1103. [PubMed: 25336726]
37. Fisher N, Abd Majid R, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neill PM, Ward SA, et al. Cytochrome b Mutation Y268S Conferring Atovaquone Resistance Phenotype in Malaria Parasite Results in Reduced Parasite bc1 Catalytic Turnover and Protein Expression. *J. Biol. Chem.* 2012; 287(13):9731–9741. [PubMed: 22282497]
38. Nam T-G, McNamara CW, Bopp S, Dharia NV, Meister S, Bonamy GMC, Plouffe DM, Kato N, McCormack S, Bursulaya B, et al. A Chemical Genomic Analysis of Decoquinone, a *Plasmodium falciparum* Cytochrome b Inhibitor. *ACS Chem. Biol.* 2011; 6(11):1214–1222. [PubMed: 21866942]
39. Dong CK, Urgaonkar S, Cortese JF, Gamo F-J, Garcia-Bustos JF, Lafuente MJ, Patel V, Ross L, Coleman BI, Derbyshire ER, et al. Identification and Validation of Tetracyclic Benzothiazepines as *Plasmodium falciparum* Cytochrome bc1 Inhibitors. *Chem. Biol.* 2011; 18(12):1602–1610. [PubMed: 22195562]
40. Freije WA, Mandal S, Banerjee U. Expression Profiling of Attenuated Mitochondrial Function Identifies Retrograde Signals in *Drosophila*. *G3: Genes, Genomes, Genet.* 2012; 2(8):843–851.
41. Taylor CM, Wang Q, Rosa BA, Huang SC-C, Powell K, Schedl T, Pearce EJ, Abubucker S, Mitreva M. Discovery of Anthelmintic Drug Targets and Drugs Using Chokepoints in Nematode Metabolic Pathways. *PLoS Pathog.* 2013; 9(8):e1003505. [PubMed: 23935495]
42. Huang SC-C, Freitas TC, Amiel E, Everts B, Pearce EL, Lok JB, Pearce EJ. Fatty Acid Oxidation Is Essential for Egg Production by the Parasitic Flatworm *Schistosoma mansoni*. *PLoS Pathog.* 2012; 8(10):e1002996. [PubMed: 23133378]
43. Seeber F, Limenitakis J, Soldati-Favre D. Apicomplexan mitochondrial metabolism: a story of gains, losses and retentions. *Trends Parasitol.* 2008; 24(10):468–478. [PubMed: 18775675]
44. Foth BJ, Stimmer LM, Handman E, Crabb BS, Hodder AN, McFadden GI. The malaria parasite *Plasmodium falciparum* has only one pyruvate dehydrogenase complex, which is located in the apicoplast. *Mol. Microbiol.* 2005; 55(1):39–53. [PubMed: 15612915]
45. Woehrer W, Roehr M. Regulatory aspects of bakers' yeast metabolism in aerobic fed-batch cultures. *Biotechnol. Bioeng.* 1981; 23(3):567–581.
46. Hall N, Karras M, Raine JD, Carlton JM, Kooij TWA, Berriman M, Florens L, Janssen CS, Pain A, Christophides GK, et al. A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science.* 2005; 307(5706):82–86. [PubMed: 15637271]
47. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, De La Vega P, Holder AA, Batalov S, Carucci DJ, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science.* 2003; 301(5639):1503–1508. [PubMed: 12893887]
48. Tarun AS, Peng X, Dumpit RF, Ogata Y, Silva-Rivera H, Camargo N, Daly TM, Bergman LW, Kappe SHI. A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105(1):305–310. [PubMed: 18172196]

49. Hino A, Hirai M, Tanaka TQ, Watanabe YI, Matsuoka H, Kita K. Critical roles of the mitochondrial complex II in oocyst formation of rodent malaria parasite *Plasmodium berghei*. J. Biochem. 2012; 152(3):259–268. [PubMed: 22628552]
50. Sturm A, Mollard V, Cozijnsen A, Goodman CD, McFadden GI. Mitochondrial ATP synthase is dispensable in blood-stage *Plasmodium berghei* rodent malaria but essential in the mosquito phase. Proc. Natl. Acad. Sci. U. S. A. 2015; 112(33):10216–10223. [PubMed: 25831536]
51. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 1979; 65(3):418–420. [PubMed: 383936]
52. Boysen KE, Matuschewski K. Arrested Oocyst Maturation in *Plasmodium* Parasites Lacking Type II NADH:Ubiquinone Dehydrogenase. J. Biol. Chem. 2011; 286(37):32661–32671. [PubMed: 21771793]

Figure 1.

A putative model of the TCA cycle and the ETC of the *Plasmodium* mitochondrion. Substrates and inhibitors used in this study are indicated in bold blue and red text, respectively. The localization of NDH2 to either the matrix or the intermembrane space side remains a matter of debate.⁵² Abbreviations: CytC ox, oxidizing form of cytochrome *c*; CytC red, reducing form of cytochrome *c*; G3PDH, glycerol-3-phosphate dehydrogenase; NDH2, NADH:ubiquinone oxidoreductase; DHODH, dihydroorotate dehydrogenase; SDH, succinate:ubiquinone oxidoreductase; MQO, malate:quinone oxidoreductase; Q, ubiquinone; QH₂, ubiquinol; Qo, ubiquinol oxidation site; and Qi, ubiquinone reduction site.

Figure 2.

XFe24 analyzer enables the monitoring of glycolysis and mitochondrial respiration in *P. falciparum*. (a) iRBCs show robust ECAR but not OCR. iRBCs were obtained by MACS column (Miltenyi Biotec), and OCR and ECAR values were measured for iRBCs, a 1:1 mixture of iRBCs and uRBCs, and uRBCs (1 million cells/well). Data represent means $\pm$ SD ($n = 7$), and one representative analysis of two bioreplicates is shown. (b) Kinetic ECAR readouts represent glycolytic activity. Saponin-freed schizonts were exposed to a glycolysis inhibitor, 2-deoxyglucose (2-DG, 100 μ M), or medium as a control. The spike observed in

the kinetic OCR graph is explained as a response to the ATP loss due to glycolysis inhibition. Data represent means $\pm$ SD ($n = 3$), and one representative analysis of two bioreplicates is shown. (c) Kinetic OCR readouts represent inhibition of complex III and restoration of electron flow by chemical reduction. Saponin-freed schizonts were exposed to antimycin A (AA, 1 μ M) and then a mixture of TMPD (0.3 mM) and ascorbate (A, 0.5 mM), or medium, as a control. Data represent means $\pm$ SD ($n = 3$), and one representative analysis of three bioreplicates is shown. (d) Optimization of seeding density for schizonts. Saponin-freed parasites were seeded at six different densities (26.8-, 18.3-, 13.4-, 9.4-, 6.7-, and 4.7-million cells/well), and OCR and ECAR were measured. Ten to twelve million cells/well seem to be the ideal seeding density for OCR and ECAR analyses. Data represent means $\pm$ SD ($n = 3$), and one representative analysis of three bioreplicates is shown. All experiments were conducted in unbuffered RPMI.

Figure 3.

Saponin-free schizonts remain bioenergetically active in MAS. (a) Kinetic OCR readouts in three different assay media. Freed schizonts were seeded in MAS, MAS supplemented with glucose (15 mM), or MAS supplemented with succinate (10 mM). OCR and ECAR were monitored for approximately 1 h. Antimycin A (0.5 μ M) was added to confirm that the observed oxygen consumption is attributed to mitochondrial respiration. ECAR (300 pmol/min) was only detectable in MAS containing glucose. One representative analysis of two bioreplicates is shown. (b) ECAR and OCR induction by glucose can be observed for

approximately 100 min. Parasites were exposed to glucose (2 mM) at three different time points (T_1 , T_2 , or T_3) in MAS. For the “pre-treat” condition, glucose was added just before the assay plate was loaded to the XFe24 analyzer, and then the reading started after the equilibration period (12 min). One representative analysis of two bioreplicates is shown. (c) Bioenergetic profiles of parasites at various stages. Parasites at ring, trophozoite, or schizont stages were exposed to glucose (2 mM) in MAS, and the increase in OCR and ECAR was measured approximately 30 min later. All data represent means $\pm$ SD ($n = 3$).

Figure 4.

Various mitochondrial substrates can induce oxygen consumption in freed schizonts. (a) Addition of malate, but not pyruvate, induces OCR increase. Medium, malate, or pyruvate was repeatedly added to parasites at the four dotted lines. Nontreated (medium) parasites exhibited OCR that decreased slowly over the experimental period. The malate-treated parasites had increased OCR at every addition; in contrast, the pyruvate-treated parasites showed a similar OCR response as nontreated parasites. Data represent means $\pm$ SD ($n = 3$), and one representative analysis of two bioreplicates is shown. (b–f) Titration of

mitochondrial substrates to determine optimal concentrations for OCR induction. Parasites were exposed to six different concentrations of test compounds, and the average OCR values of three readouts (within 12 min) before and after compound addition were calculated. OCR increase was determined as the difference of these two values. Data represent mean $\pm$ SEM ($n = 6-10$) of three to five bioreplicates, and the determined optimal concentration is highlighted. (g) Comparison of the maximum OCR achieved by succinate, malate, glutamate, DHO, and G3P. The test compounds were added to parasites at the determined optimal concentrations, and average OCR values of three readouts (within 12 min) before and after the compound addition were calculated. OCR increase was determined as the difference of these two values, and the experiment was repeated twice. Data represent mean $\pm$ SEM ($n = 6$) of two bioreplicates. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ compared with 0 mM (b-f) or G3P (g).

Figure 5.

Malate increases OCR in fully permeabilized parasites in contrast to glucose and pyruvate. (a) Effect of digitonin titration on OCR and ECAR. Freed schizonts were exposed to different concentrations of digitonin in MAS supplemented with glucose (6 mM), and OCR and ECAR values were measured approximately 70 min later. Parasites without digitonin treatment (0 μ M) were subsequently exposed to antimycin A (1 μ M), and OCR and ECAR values were measured. Data represent means $\pm$ SD ($n = 3$), and one representative analysis of two bioreplicates is shown. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ compared with

0 μ M (OCR). ⁺⁺⁺⁺, $P = 0.0001$ compared with 0 μ M (ECAR). (b) OCR induction in freed schizonts with or without digitonin treatment. Parasites were first treated with digitonin (300 μ M) in MAS for approximately 30 min and then exposed to control (medium), glucose (1 mM), malate (40 mM), or pyruvate (40 mM). Parasites without digitonin treatment were also exposed to medium, glucose (1 mM), or malate (40 mM) with the same timing. The changes of OCR values 15 min later is shown. Data represent means $\pm$ SD ($n = 3$, parasites with digitonin treatment; $n = 2$, parasites without digitonin treatment), and one representative analysis of two bioreplicates is shown. *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$ compared with medium.

Figure 6.

Glucose titration reveals metabolic shifts between mitochondrial respiration and glycolysis. (a) OCR and ECAR peaks at different concentrations of glucose. Freed schizonts were exposed to six different glucose concentrations in MAS, and OCR and ECAR were measured 35 min later. Data represent mean $\pm$ SEM of three bioreplicates ($n = 6$). **, $P < 0.01$; ****, $P < 0.0001$ compared with 0 μ M (OCR). ++, $P < 0.01$; +++, $P < 0.0001$ compared with 0 μ M (ECAR). (b) Kinetic OCR and ECAR readouts in response to various glucose concentrations. Freed schizonts in MAS were exposed to glucose at five different

concentrations, followed by antimycin A (1 μ M). Data represent means $\pm$ SD ($n = 3$), and one representative analysis of three bioreplicates is shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 7.

Freed schizonts exhibit low OXPHOS and high proton leak. (a) Titration of ADP to determine optimal concentrations for OCR induction. Parasites were exposed to seven different concentrations of ADP for approximately 30 min and then antimycin A (1 μ M) in unbuffered RPMI. OCR values before antimycin A addition were normalized and compared (100%: OCR values before ADP addition; 0%: OCR values after antimycin A treatment). Data represent means $\pm$ SEM ($n = 6$) of two bioreplicates. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ compared with 0 μ M. (b) Oligomycin A reduced ADP-induced OCR in a dose-dependent manner. Parasites were exposed to different concentrations of oligomycin A or antimycin A (1 μ M) in unbuffered RPMI supplemented with ADP (24 mM) (100%: OCR values before compound addition; 0%: OCR values of antimycin A treatment). (c) OCR fluctuation by sequential exposure to ADP (24 mM), oligomycin A (10 μ M), FCCP (2 μ M), and antimycin A (1 μ M) in unbuffered RPMI. Data represent means $\pm$ SD ($n = 4$), and one representative analysis of two bioreplicates is shown. (d) Comparison of OCR levels induced by the compounds used in (c). All compounds were added to parasites simultaneously in unbuffered RPMI, and average OCR values of three readouts (within 12 min) before and after the compound addition were compared. Data represent means $\pm$ SD ($n = 3$), and one

representative analysis of two bioreplicates is shown. *, $P = 0.05$; ****, $P = 0.0001$ compared with medium.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8.

Dose-responsive OCR reduction is observed with cytochrome *b* inhibitors: antimycin A, atovaquone, IDI-5918, BTZ-1, and decoquinatate. Freed schizonts were exposed to three doses of test compounds or antimycin A (1 μ M) in unbuffered RPMI (100%: OCR values before compound addition; 0%: OCR values of antimycin A treatment). All data represent means $\pm$ SD ($n = 3$), and one representative analysis of two or three bioreplicates is shown.

Figure 9.

OCR readouts can be used for target identification and mode of action study of mitochondrial inhibitors. (a) DHODH inhibition by genz-669178 is not observable in unbuffered RPMI. Freed schizonts were exposed to genz-669178 (1 μ M) or antimycin A (1 μ M) for 45 min. (b) Freed schizonts were exposed to one of the following substrates: DHO (80 mM), succinate (40 mM), malate (40 mM), or G3P (8 mM) in MAS. Then, genz-669178 (1 μ M) and antimycin A (1 μ M) were sequentially added to all parasites. Genz-669178 only reduced the DHO-induced OCR increase, whereas antimycin A reduced OCR in all conditions. (c) Cytochrome *b* inhibitors disrupt the electron flow of the ETC in transgenic *scDHODH*-expressing parasites. Freed schizonts were exposed to antimycin A (1 μ M), atovaquone (1 μ M), BTZ-1 (1 μ M), or medium in unbuffered RPMI for 45 min. Note: The different profiles of OCR reduction seen with different inhibitors are likely due to differences in the compounds' binding sites and chemical properties, such as solubility and permeability. All data represent means $\pm$ SD ($n = 3$), and one representative analysis of two bioreplicates is shown.