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A high-throughput fluorescence-based assay for *Plasmodium* dihydroorotate dehydrogenase inhibitor screening

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ABSTRACT

Plasmodium dihydroorotate dehydrogenase (DHODH) is a mitochondrial membrane-associated flavoenzyme that catalyzes the rate-limiting step of de novo pyrimidine biosynthesis. DHODH is a validated target for malaria, and DSM265, a potent inhibitor, is currently in clinical trials. The enzyme catalyzes the oxidation of dihydroorotate to orotate using flavin mononucleotide (FMN) as cofactor in the first half of the reaction. Reoxidation of FMN to regenerate the active enzyme is mediated by ubiquinone (CoQ_D), which is the physiological final electron acceptor and second substrate of the reaction. We have developed a fluorescence-based high-throughput enzymatic assay to find DHODH inhibitors. In this assay, the CoQ_D has been replaced by a redox-sensitive fluorogenic dye, resazurin, which changes to a fluorescent state on reduction to resorufin. Remarkably, the assay sensitivity to find competitive inhibitors of the second substrate is higher than that reported for the standard colorimetric assay. It is amenable to 1536-well plates with Z' values close to 0.8. The fact that the human enzyme can also be assayed in the same format opens additional applications of this assay to the discovery of inhibitors to treat cancer, transplant rejection, autoimmune diseases, and other diseases mediated by rapid cellular growth.

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Malaria is caused by five species of apicomplexan parasites of the genus *Plasmodium* that affect humans. The most deadly form is caused by *P. falciparum* and predominates in Africa, whereas *P. vivax* is less dangerous but more widespread. In 2013, 198 million cases were estimated to have occurred globally, and the disease killed 367,000 to 755,000 people [1], with children under 5 years of age and pregnant women being most severely affected. Resistance to artemisinins—the key compounds in artemisinin-based combination therapies—has been detected in five countries of Southeast Asia: Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand, and Viet Nam. Although such resistance has not yet led to operational failure of malaria control programs, urgent and intensified efforts are required to prevent a future public health disaster and new and differentiated treatments are needed.

Several antimalarial drugs in clinical studies target pyrimidine nucleotide biosynthesis [2,3] because *Plasmodium* parasites rely on fast and large replication of DNA to infect during liver and blood stages. Dihydroorotate dehydrogenase (DHODH) catalyzes the oxidation of L-dihydroorotate (L-DHO) to L-orotate in the fourth step in the de novo pyrimidine biosynthetic pathway. It is essential for *Plasmodium* species survival because, unlike humans, malaria parasites are unable to scavenge preformed pyrimidines [4]. This is the only redox and rate-limiting step in uridine monophosphate (UMP) formation, the precursor to all the other pyrimidines used to synthesize DNA, RNA, and various cofactors [5].

Plasmodium falciparum DHODH (*Pf*DHODH) belongs to family 2, found in gram-negative bacteria and eukaryotes. The enzyme is attached to the inner mitochondrial membrane and contains a tightly bound flavin mononucleotide (FMN) cofactor that is reduced on L-DHO oxidation to L-orotate in the first half of the reaction cycle. This cofactor is recycled to its oxidized form in the second half of the reaction, transferring the electrons to the ubiquinone (CoQ_D) that acts as natural final electron acceptor, chemically coupling pyrimidine biosynthesis to the respiratory chain [6].

Abbreviations used: DHODH, dihydroorotate dehydrogenase; L-DHO, L-dihydroorotate; *Pf*DHODH, *Plasmodium falciparum* DHODH; FMN, flavin mononucleotide; CoQ_D, ubiquinone; mETC, mitochondrial electron transport chain; DCIP, 2,6-dichloroindophenol; HTS, high-throughput screening; GSK, GlaxoSmithKline; FLINT, fluorescence intensity; HsDHODH, *Homo sapiens* (human) DHODH; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-L-propanesulfonate; tdBSA, thermally denatured bovine serum albumin; β-ME, β-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; CMC, critical micelle concentration.

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One key function of the parasite mitochondrion is to maintain the mitochondrial electron transport chain (mETC) to regenerate the CoQ_D required as electron acceptor for *Pf*DHODH. This is demonstrated by the fact that parasites are very sensitive to mETC inhibitors, but transgenic strains expressing ubiquinone-nondependent DHODH from *Saccharomyces cerevisiae* are resistant to them. These results provide a genetic validation of *Plasmodium* DHODH as an attractive antimalarial target [7].

Potent inhibitors of the human enzyme, such as lapachol, brequinar, and leflunomide, are poorly active against *Pf*DHODH. Thus, these data suggest that it should be feasible to exploit active-site differences to identify inhibitors that exhibit a high degree of selectivity toward malarial DHODH [8]. The sequence of the L-DHO binding site is highly conserved, but the sequence of the quinone-binding N-terminal domain is variable [9]. This variability is thought to be responsible for the high degree of species-related preferential inhibition observed among DHODH family 2 members. So, therapeutic agents, both those targeted to rapidly proliferating human cells and those targeted to human pathogens, could be designed to explicitly exploit these differences.

DHODH activity has been traditionally measured with the standard colorimetric assay that monitors 2,6-dichloroindophenol (DCIP) reduction as absorbance decrease at 600 nm [10]. This assay has permitted the identification of several families of *Pf*DHODH inhibitors in a successful high-throughput screening (HTS) campaign of a chemical library containing 220,000 compounds in 384-well plates and 50 μl final volume [11]. The optimization of the initial hits resulted in the identification of DMS265 [12]. This molecule is a potent, first in class inhibitor of *Pf*DHODH with an in vivo potency similar to chloroquine, and has been recently progressed to clinical studies in phase 2 [13]. This fact has renewed the interest in finding new *Pf*DHODH inhibitors, and GlaxoSmithKline (GSK) has accomplished the screen of a compound collection of 1.5 million in HTS format. To achieve this task, we started by trying to miniaturize the colorimetric assay to 10 μl in a 1536-well format, but we found it to be not robust enough. Colorimetric assay conditions include the presence of detergent to solubilize the quinone substrate, glycerol to stabilize the enzyme, and sodium dodecyl sulfate (SDS) to stop the reaction. Preliminary trials were unsuccessful because buffer components made the mixing steps hard, resulting in nonreproducible dispensations, and formation of bubbles disturbed the absorbance reading in 1536-well plates. Thus, we explored the possibility of developing a fluorescence assay to make it more amenable to ultra-high-throughput mode.

Here we report the development of a new fluorescence intensity (FLINT), signal increase, high-throughput assay that measures the oxidation of L-DHO to L-orotate by DHODH. In this reaction, the reducing equivalents from the oxidation of the L-DHO are transferred to the FMN, yielding the reduced enzyme form that, in the second-half reaction, becomes reoxidized by an electron acceptor. In the physiological environment, the final acceptor, and the second substrate of the reaction, is the respiratory ubiquinone, CoQ_D. In our assay, the CoQ_D has been replaced by resazurin, a redox-sensitive fluorogenic dye that changes from a blue nonfluorescent state to a pink highly fluorescent state on reduction to resorufin (Fig. 1). In the standard colorimetric assay, the ubiquinone is present in the reaction, and electrons are transferred from CoQ_D to the final artificial acceptor DCIP. It is reported the DCIP acts as alternative substrate to CoQ_D and the observed activity is the sum of the reduction of both the quinone and the dye [14]. Under typical assay conditions, and depending on the quinone substrate, 10–30% of the observed activity is due to the direct reduction of DCIP by DHODH. The presence of two substrates, one of them silent, complicates inhibition experiments and leads to errors if both substrates are not explicitly considered in analysis [15].

Alternative assay formats have been used to simplify data interpretation in the mechanism of inhibition studies such as colorimetric quantification of L-orotate, ferricyanide, and FMN fluorescence transitions [6,15]. In the FLINT assay described here, the quinone is removed and substituted by resazurin because the combined addition of both substrates, silent quinone and resazurin, decreased resorufin production and sensitivity to inhibition. Doing so, the assay increases its sensitivity to find inhibitors that compete for the quinone binding site. We report the kinetic parameters and optimal assay conditions to test *Plasmodium* and human (*Homo sapiens*) DHODH (*Hs*DHODH) activities in a FLINT ultra-high-throughput format that allows the detection of inhibitors.

Materials and methods

Materials

All reagents were obtained from either Merck Millipore or Sigma–Aldrich of the highest purity available except resazurin sodium salt from Invitrogen. *N*-(3-Bromo-phenyl)-2-methyl-3-nitro-benzamide (compound **1**), *N*-(3,5-dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound **2**), and *N*-(3,4-difluoro-phenyl)-2-methyl-3-nitro-benzamide (compound **3**) were kindly provided by Jon Clardy (Harvard Medical School).

Enzymes

*Pf*DHODH and *Hs*DHODH enzymes were kindly provided by Jon Clardy. Plasmids encoding *Pf*DHODH (pET20b-pfDHODH (158–569)-TEV) and *Hs*DHODH (pET28a-10H-EK-hDHODH (29–235)) were used to transform BL21STAR (DE3) *Escherichia coli* competent cells. Protein expression and purification has already been reported [9,16].

Kinetic characterization

To assess the capacity of *Pf*DHODH to use resazurin as substrate, 150 nM *Pf*DHODH was incubated with 100 μM resazurin and 50 μM DHO in the presence and absence of 100 μM CoQ_D in a buffer containing 200 nM FMN, 0.05% Triton X-100, and 100 mM Hepes (pH 8.0, 20 μl final volume) in a 384-well plate. Resorufin production was quantified kinetically at room temperature as FLINT emission increase at 590 nm using 555 nm for excitation and a cutoff at 570 nm with a Gemini SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). To elucidate the enzymatic mechanism, 20 nM *Pf*DHODH was incubated against a grid titration of L-DHO and resazurin. Initial velocities were fitted with rate equations described for a two-site ping-pong mechanism (double–displacement reaction), which can be found elsewhere [17]. To determine the k_{cat} and K_M for the enzymes in the final assay conditions, several concentrations of the substrates (L-DHO and resazurin for fluorescence assay; L-DHO and CoQ_D for colorimetric assay) were incubated at apparent saturating concentrations of the other (1 mM L-DHO, 200 μM resazurin, and 60 μM CoQ_D). Enzyme concentrations were 20 and 50 nM *Pf*DHODH for FLINT and colorimetric assays, respectively, and 5 and 7.5 nM for *Hs*DHODH. Buffer contained 150 mM NaCl, 5% glycerol, and either 0.1% Triton X-100 or 5 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-L-propanesulfonate) in 100 mM Hepes (pH 8.0). Data were fitted with a Michaelis–Menten equation using GraFit (Erithacus Software, Horley, UK).

Assay optimization

Effect of additives. To study the effect of several chemicals on enzyme activity, different reagents were added to reaction mixtures containing 40 nM *Pf*DHODH, 50 μM L-DHO, and 50 μM

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