UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Repurposing of antimalarial drugs in the treatment of schistosomiasis based on the selective inhibition of the enzyme dihydroorotate dehydrogenase

Reposicionamento de drogas antimaláricas no tratamento da esquistossomose baseado na inibição seletiva da enzima diidroorotato desidrogenase

Felipe Antunes Calil

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Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

Concentration Area: Chemistry and Biological Physics

Supervisor: Prof. Dr. Maria Cristina Nonato

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Inscription

A minha esposa, Anelize, que com seu carinho e intenso incentivo, acreditou na minha capacidade e me ensinou a amar a pesquisa. Sem você este trabalho não existiria.

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“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth”

Jules Verne, A Journey to the Center of the Earth

“Words are, in my not-so-humble opinion, our most inexhaustible source of magic, capable of both influencing injury, and remedying it.”

J.K. Rowling, Harry Potter and the Deathly Hallows

RESUMO

CALIL, FELIPE. A. **Reposicionamento de drogas antimaláricas no tratamento da esquistossomose baseado na inibição seletiva da enzima diidroorotato desidrogenase**. 2018. 135f. Tese de Doutorado. Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2018.

Reposicionamento de fármacos, a aplicação de um tratamento existente a uma nova doença, promete um rápido impacto clínico a um custo menor do que o desenvolvimento de novos fármacos. Essa estratégia é de particular interesse para o desenvolvimento de novos fármacos para o tratamento de doenças negligenciadas, devido à escassez de investimento e ao elevado número de indivíduos afetados. A esquistossomose é uma doença crônica debilitante causada por trematódeos do gênero *Schistosoma* que afeta mais de 200 milhões de pessoas no mundo. Apesar de ser um grave problema de saúde, o único medicamento disponível, Praziquantel, está se tornando um grave problema devido à resistência parasitária. Neste contexto, este trabalho teve como objetivo avaliar compostos sintéticos que apresentam atividade antimalárica para potencial descoberta de novas drogas contra a esquistossomose. Os compostos foram selecionados com base em sua capacidade de inibir a enzima diidroorotato desidrogenase (DHODH). A enzima DHODH participa da síntese de novo dos nucleotídeos de pirimidina e é um alvo terapêutico validado para muitas doenças. Nossos estudos identificaram vários compostos como potentes inibidores da diidroorotato desidrogenase (*Sm*DHODH) do *S. mansoni*. Um total de 34 compostos foram identificados como inibidores e estudos mecanísticos permitiram agrupá-los em três classes: inibidores competitivos, não competitivos e misto. Os estudos inibitórios, juntamente com ensaios de estabilidade térmica, sugerem que não apenas a distribuição química e estérica do sítio de ligação é importante, mas a dinâmica do domínio N-terminal desempenha um papel importante na interação do ligante. O índice de seletividade (SI) foi estimado pela avaliação dos melhores acertos contra a enzima homóloga humana (*Hs*DHODH). Os resultados identificaram o composto 17 (2-hidroxi-3-isopentilnaftaleno-1,4-diona) como o melhor composto para a inibição seletiva de *Sm*DHODH (IC_{50} = 23 ± 4 nM, SI 30,83). Estudos *in vitro* utilizando vermes adultos de *S. mansoni* foram usados para identificar o impacto de compostos selecionados na morfologia e atividade esquistossomicida. Os resultados mostram uma atividade potente contra os parasitas, especialmente para o composto atovaquona, um antimalárico.
O *Plasmodium falciparum* DHODH (*Pf*DHODH), um alvo validado contra a malária, também foi foco do presente trabalho. Desenvolvemos um pipeline para avaliar a potência, seletividade e mecanismo de inibição. Em nosso trabalho, diferentes classes de compostos foram testadas e os ligantes identificados tiveram seu mecanismo de inibição determinado. A cristalização de *Pf*DHODHΔloop (onde o loop flexível de Gly^{285} até Lys^{294} foi removido) foi alcançada com sucesso e fornecerá a base estrutural para entender a potência e seletividade de ligantes. Nossos resultados apoiam nossa proposta original de reaproveitar compostos e/ou seus análogos, originalmente desenvolvidos contra o *Pf*DHODH, para buscar estratégias alternativas para o tratamento da esquistossomose.

Palavras-chave: Esquistossomose, diidroorotato desidrogenase, reposicionamento de drogas, doenças tropicais negligenciadas, malária

ABSTRACT

CALIL, FELIPE. A. **Repurposing of antimalarial drugs in the treatment of schistosomiasis based on the selective inhibition of the enzyme dihydroorotate dehydrogenase**. 2018. 135f. Doctoral Thesis. Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2018.

Drug repurposing, the application of an existing therapeutic to a new disease indication, holds the promise of rapid clinical impact at a lower cost than *de novo* drug development. This strategy is of particular interest for the development of new drugs for the treatment of neglected diseases, due to the scarcity of investment and the high number of individuals affected. Schistosomiasis is a chronic, debilitating disease caused by trematodes of the genus *Schistosoma* affecting over 200 million people worldwide. Despite being a serious health burden, the only drug available, Praziquantel, is becoming a significant issue due to parasite resistance. In this context, this work aimed to evaluate synthetic compounds which display antimalarial activity as potential leads for the discovery of new therapeutics for schistosomiasis. The compounds were selected based on their ability to inhibit the enzyme dihydroorotate dehydrogenase (DHODH). DHODH enzyme participates in the *de novo* synthesis of pyrimidine nucleotides and it is a validated therapeutic target for many diseases. Our studies identified several compounds as potent inhibitors of *Schistosoma mansoni* dihydroorotate dehydrogenase (*Sm*DHODH). A total of 34 compounds were identified as inhibitors and mechanistic studies allowed us to sort them into three classes: competitive, non-competitive and mixed-type inhibitors. The inhibitory studies together with thermal stability assays suggest that not only chemical and steric distribution of the binding pocket is important but dynamics of the N-terminal helical domain plays an important role in ligand binding. The selectivity index (SI) was estimated by evaluating the best hits against the human homologue enzyme (*Hs*DHODH). The results identified compound 17 (2-hydroxy-3-isopentylnaphthalene-1,4-dione) as the best compound for *Sm*DHODH selective inhibition (IC_{50} = 23 ± 4 nM, SI 30.83). *In vitro* studies using adult *S. mansoni* worms were used to identify the impact of selected compounds on the morphology and schistosomicidal activity. Results show a potent activity against the parasites, especially for the compound atovaquone, an antimalarial drug.
Plasmodium falciparum DHODH (*Pf*DHODH), a validated target against malaria, was also focus of the present work. We developed a pipeline to evaluate potency, selectivity and mechanism of inhibition. In our work, different classes of compounds were assayed and identified ligands had their mechanism of inhibition determined. Crystallization of *Pf*DHODHΔloop (where the flexible loop from Gly^{285} to Lys^{294} was removed) was successfully achieved and will provide the structural basis to understand potency and selectivity of ligands. Our results support our original proposal of repurposing compounds and/or its analogues, originally developed against *Pf*DHODH, to search for alternative strategies to treat schistosomiasis.

Keywords: Schistosomiasis, dihydroorotate dehydrogenase, drug repositioning, neglected tropical diseases, malaria

CHAPTER 1. INITIAL CONSIDERATIONS

1.1 General Introduction

On the bright side, globalization can be considered a set of processes that afford easy access to knowledge, economic stability and social equality.[1] However, it has also helped to spread some of the deadliest infectious diseases across wide geographic areas. Malaria has crossed the African borders and became global as humans migrated to other continents and parasites got adapted to different mosquito species that were evolutionarily distant from African vectors.[2] Ebola virus outbreak, which killed more than 11,000 people and infected at least 28,000, is a stark reminder of the world fragility in health security.[3] A recent example of the negative impact of globalization is the spread of Zika virus from Uganda in 1947,[4, 5] to other African countries within a few years, and then to the Micronesia, to finally arrive at Americas late December 2015, where 440,000–1,300,000 suspected cases have occurred.[6] Despite the fact that Zika has drawn attention to flavivirus infections which remained largely forgotten by the pharmaceutical industry, the scenario for other neglected diseases remains unchanged.

The Neglected Tropical Diseases (NTDs) consist of a large number of diseases (e.g. Chagas disease, dengue, leishmaniasis, malaria, leprosy, schistosomiasis, among others) caused by several pathogens such as viruses, bacteria, protozoa and helminths. These diseases together affect more than 1 billion people, including half a billion children, especially in poor and marginalized areas, representing a serious burden to public health.[7, 8] To make matters worse, NTDs, previously found in developing countries, are also becoming widespread posing a serious challenge to the health systems of developed countries that receive immigrants and refugees from endemic areas.[9, 10]

Although the tropical diseases are a major cause of morbidity and mortality in the world, the lack of investment in research and development of new therapies is still a striking feature, as well as the resistance of pathogens to the already existing drugs.[11] Thus, NTDs are a global public health problem, and new policies for control, prevention and investment in research and development, both by the public and private sectors, are essential for the implementation of new diagnostic strategies and effective therapeutic modalities.

Many steps are necessary to ensure efficacy and safety of a drug before being approved to patient's uses. In the best case scenario, the average cost to introduce a drug in the market is about U$ 800,000,000, that in a period of 12 to 15 years. In average, out of ten thousand compounds identified and submitted to pre-clinical assays, only five are approved to clinical trials, from which only one is approved to clinical use (**Figure 1**).[12]

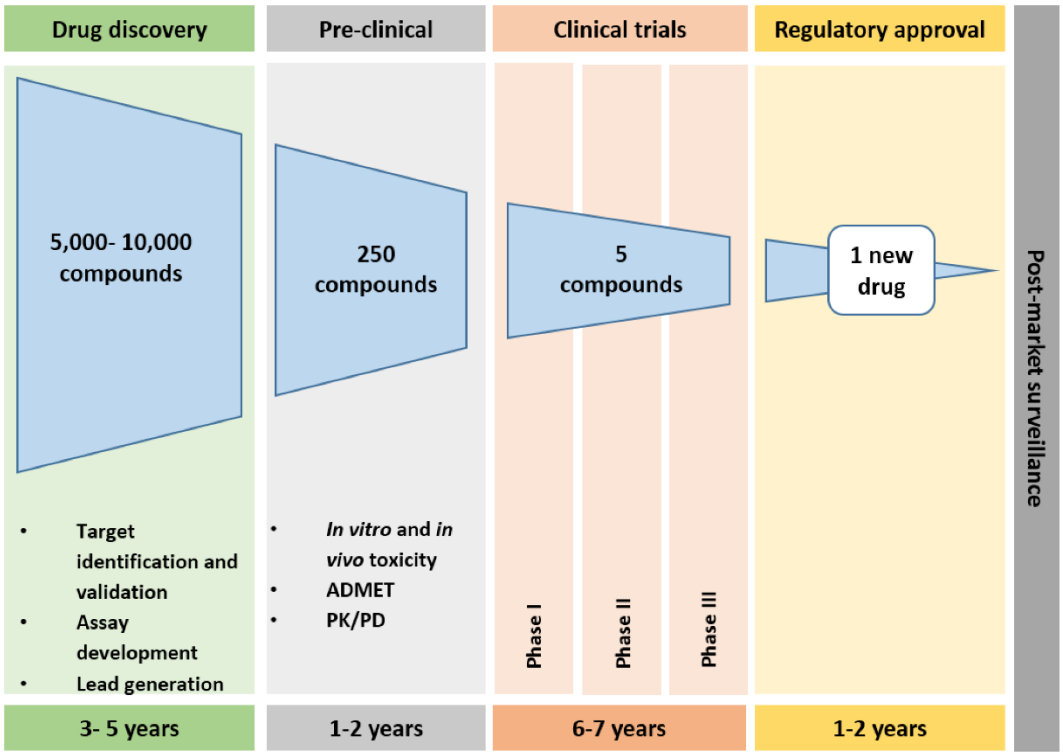

Figure 1. Drug discovery and development timeline. The current drug approval pipeline can take up to 15 years. It is estimated that from 5,000–10,000 compounds only one new drug reaches the market (extracted from Matthews, Hanison and Nirmalan, 2016 [12]).

The gap between compound discovery and its use in clinical trials is called the "Valley of Death" and represents a major barrier to the development of new drugs. Among several, one of the major limitations to decrease this gap is the high cost associated with this step during the translational process.

One way to accelerate the pharmaceutical research process is through the use of a strategy called drug repositioning (or repurposing of drugs). This strategy consists on

the evaluation and/or use of existing drugs for the treatment of diseases other than those for which they were originally developed.[13] Through repositioning of drugs, it is expected that bioactive molecules will be identified within shorter development periods, besides the risk reduction with compounds that have already undergone regulatory clinical trials.[14] Previous pharmacokinetic and toxicological data provide estimates of therapeutic concentrations in the new application. In addition, regulatory procedures can be expedited, in which the applicant can rely on data from previous studies. This has worked as a stimulus to identify new activities for already known molecules.[13] All these factors contribute to significant cost savings - important in the context of diseases that afflict the less favored, such NTDs, where return on investment is marginal.[14-16]

Many drugs have already been successfully repurposed and many projects using this strategy are currently under development (**Table 1**),[17] including ongoing repurposing programs (Medicines for Malaria Venture, the Global Alliance for TB Drug Development, Drugs for Neglected Diseases, and the OSDD initiative) and completely repurposed drugs that target NTDs.[18]

Table 1. Examples of repurposed or candidate drugs for many diseases, including NTDs.[17, 18]

Drug	Originally Developed	Repurposed
All-Trans Retinoic Acid (ATRA)	Severe Acne	Leukemia
Amphotericin B	Antifungal	Leishmaniasis
Astemizole	Antihistamine	Malaria
Avermectin	River blindness and Elephantiasis	Tuberculosis
Chloroquine	Malaria	Lung Cancer
Eflornithine	Anticancer	African Sleeping Sickness
Methotrexate	Anticancer	Rheumatoid Arthritis
Miltefosine	Antineoplastic	Leishmaniasis
Phosphodiesterase-inhibitor analogues	Erectile dysfunction	African Sleeping Sickness; Chagas Disease
Ropinirole	Parkinson's Disease	Restless Leg Syndrome
Tamoxifen	Anticancer	Leishmaniasis
Thalidomide	Morning Sickness	Multiple Myeloma; Leprosy

Cases of malaria in developed countries combined to worldwide investments from some of these programs are supporting the development of new strategies to treat this parasitic disease and helped malaria to be removed from the current list of NTDs. One approach was based on the selective inhibition of the enzyme dihydroorotate dehydrogenase (DHODH) from *Plasmodium sp*., parasites responsible for human malaria.[19, 20] In fact, over the last several years several molecules have been identified as selective inhibitors of *Plasmodium falciparum* DHODH (*Pf*DHODH),[21] some receiving the investment needed to overcome the "Valley of Death" and reaching the clinical trial stages.

DHODH (EC 1.3.1.14, 1.3.1.15, 1.3.5.2 or 1.3.98.1, depending on the type) is the fourth enzyme that acts in the *de novo* biosynthesis of uridylate, the precursor of all pyrimidine nucleotides (**Figure 2**); it catalyzes the oxidation of dihydroorotate to orotate according to a ping-pong-type enzymatic mechanism.[22-24]

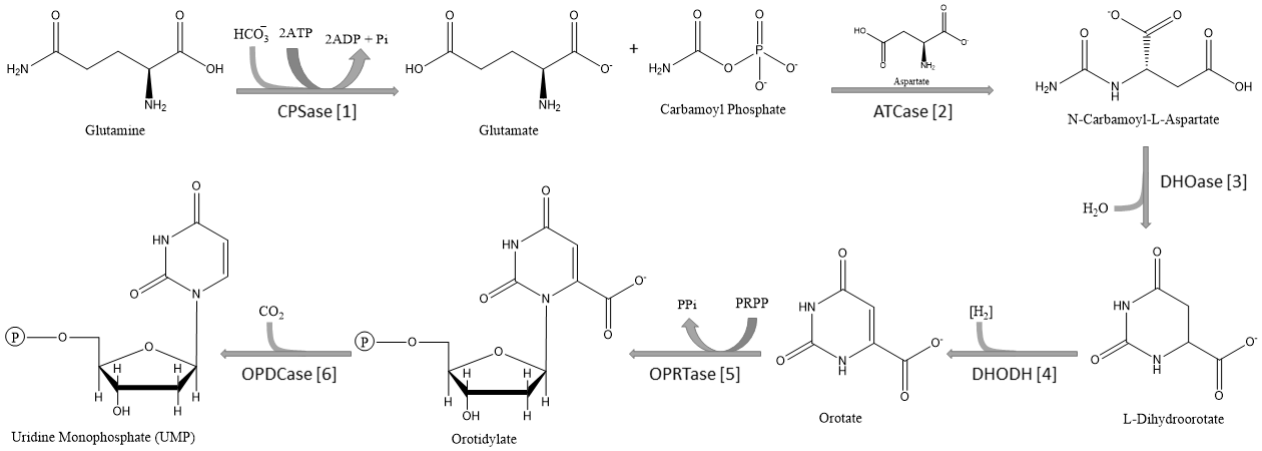

Figure 2. Schematic illustration of the pyrimidine de novo synthesis pathway.

DHODH was first detected in 1953 by Lieberman and Kornberg in extracts of the anaerobic bacterium *Zymobacterium oroticum* (now known as *Clostridium oroticum*).[25] Over the last 30 years, DHODH has been identified as the pharmacological target of a number of chemical and natural compounds such as Arava® (leflunomide, which is approved for the treatment of rheumatoid arthritis in humans), isoxazole, triazine, bicinchoninic acid and quinone derivatives.[26-28] These compounds interfere in uncontrolled reactions of the immune system, assist in fighting parasitic infections such as malaria and boost antiviral therapies by decreasing the intracellular concentration of pyrimidine nucleotides.[29] Currently, interest has arisen in exploiting DHODH inhibition as a strategy to combat a broad range of diseases,[30-37] including for malaria where the triazolopyrimidine DSM265 has progressed to clinical development.[34-38]

In the performance of its biological functions, DHODH uses flavin mononucleotide (FMN) as a cofactor. In the initial phase of the enzymatic reaction, FMN is reduced and the dihydroorotate substrate is oxidized, to orotate. In the second half of the reaction, FMN is reoxidized ($FMNH_2$ is converted to FMN) with the aid of a third molecule that acts as an electron acceptor (**Figure 3**).[33, 39]

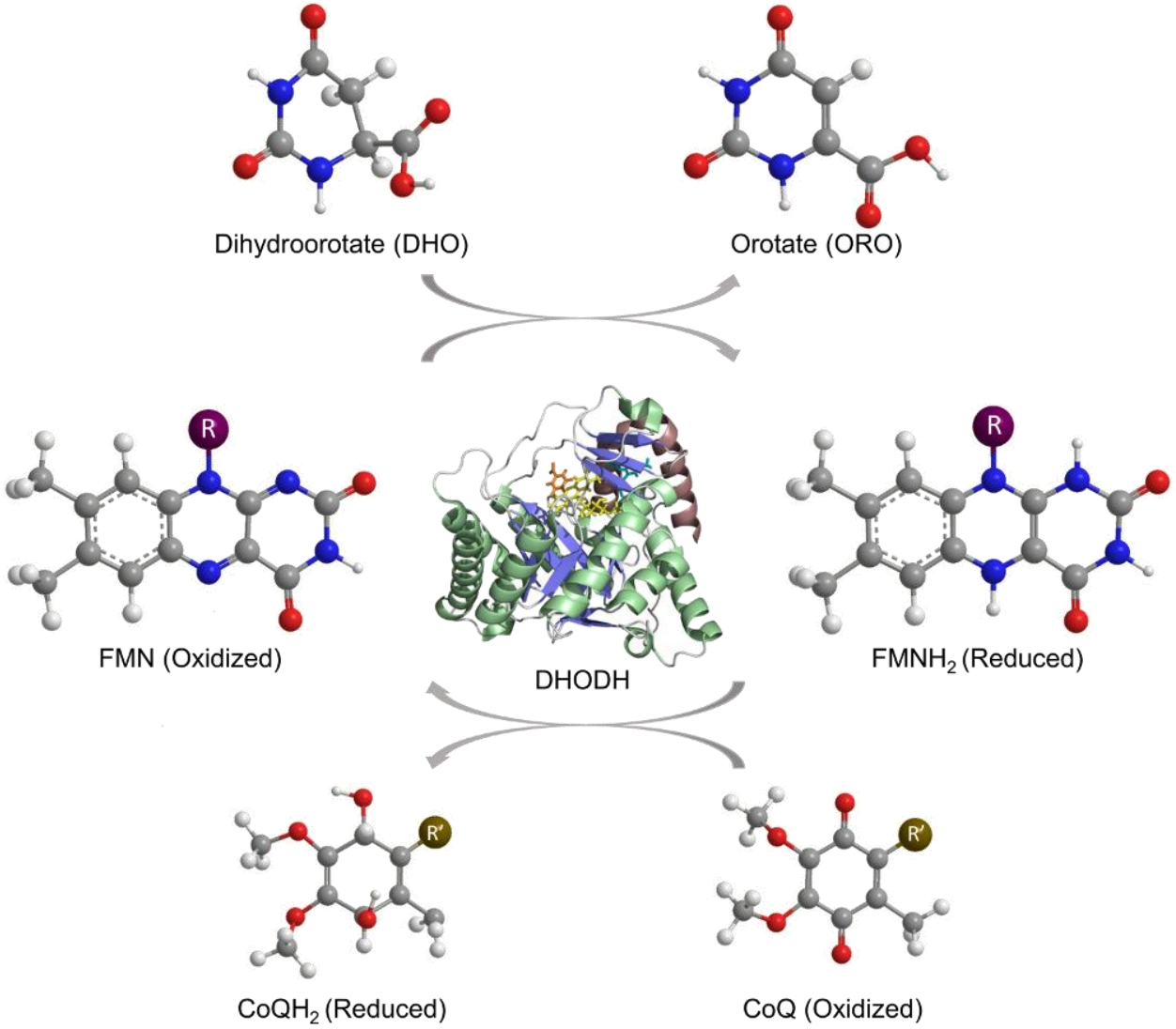

Figure 3. Enzymatic reaction catalyzed by dihydroorotate dehydrogenases (DHODHs). In this particular case, the oxidizing agent showed, CoQ, is used by Class 2 DHODHs.

According to their primary structures and cellular locations, the DHODH enzymes of various organisms can be divided into two classes, Class 1 and Class 2.[40] Class 1 enzymes can be subdivided into Classes 1A, 1B and a new class (1S) that was found in *Sulfolobus solfataricus*.[41] The Class 1 enzymes are found in the cytosol, whereas enzymes belonging to Class 2 are associated with cytosolic or mitochondrial membranes.[42, 43] Due to their association with membranes, all members of Class 2 possess an extension in the N-terminal region known as the membrane domain; this

extension allows interaction of the enzymes with the membrane.[44, 45] Enzymes belonging to Class 1 are found in Gram-positive bacteria, anaerobic fungi and in eukaryotes such as trypanosomatids. In contrast, enzymes belonging to Class 2 are found in eukaryotes and in certain prokaryotes such as Gram-negative bacteria. The division of DHODHs into two classes also correlates with the preferences of the enzymes for different electron acceptors and with their oligomeric states. The enzymes of Class 1A are homodimeric; as oxidizing agents, they use oxygen molecules or molecules that are soluble in water, such as fumarate, which oxidizes $FMNH_2$ for the regeneration of FMN. DHODHs of Class 1B are heterotetrameric enzymes that use NAD^+ as an oxidizing agent and contain not only FMN but also a flavin adenine dinucleotide molecule (FAD), besides a [2Fe-2S] cluster.[46, 47] Class 1B enzymes appear to prevail in Gram-positive bacteria, some of which express forms 1A and 1B. In contrast, the 1A form appears as a single form in selected eukaryotes, for example, in species of the genera *Leishmania* and *Trypanosoma*. Class 2 enzymes are monomeric proteins that use hydrophobic molecules (e.g., ubiquinone, CoQ_n) as oxidizing agents (**Figure 3**).[43, 48]

Another important difference between the two classes is regarding the catalytic residue, which is a cysteine for class 1, whereas a serine residue is found in members of class 2. These residues act as catalytic bases in the first step of the reaction (the reductive half-reaction), deprotonating C5 of DHO. DHO then transfer a hydride from C6 to N5 of the isoalloxazine moiety of FMN. In the second step of the reaction (the oxidative half-reaction), FMN transfer a hydride to the fumarate or the quinone, through either a direct hydride transfer, or two single-electron transfers. The mechanism for the second-half reaction is not yet fully elucidated.[24, 49]

In order to study the global reaction described above, many assays have been developed. They can be used to probe the enzymatic activity, ligand binding, and inhibition mechanisms of dihydroorotate dehydrogenase. DHODH activity can be assayed by monitoring the direct orotate formation at 300 nm ($\varepsilon = 2650\ M^{-1}\ cm^{-1}$) in a reaction mixture containing 50 mM Tris, pH 8.0, 150 mM KCl, and both substrates, dihydroorotate and oxidant agent.[22] The kinetic parameters, V_{max} and K_M, can be determined by varying the steady-state concentrations of both substrates. The reaction is initiated by the addition of

DHODH enzyme and the rate of orotate production is determined over time. Kinetic constants are estimated from the fit of the data to the equation that describes the ping-pong mechanism.[24]

With the purpose of performing the orotate formation assay described above, Triton X-100 may be used.[42, 43] Triton X-100 is typically used for screening libraries of potential inhibitors, helping solubilizing the compounds, and avoiding false positives induced by aggregate enzyme interactions, or to solubilize and stabilize class 2 DHODH enzymes. However, Triton X-100 absorbs in the near ultraviolet, interfering with the monitoring of orotate formation at 300 nm. In this situation, an indirect assay that monitors the activity of the enzyme through the use of the 2,6-dichlorophenolindophenol (DCIP) has been developed. DCIP is a colorimetric agent commonly used as the final electron acceptor in the enzymatic studies of DHODHs because the reduction of DCIP can be identified by a color change and monitored spectrophotometrically at 600-610 nm.[50-52] The reduction of DCIP is stoichiometrically equivalent to reoxidation of reduced quinone (**Figure S1A**, in appendices). This colorimetric method has been the most common assay used to monitor DHODH enzymatic reactions. It has been widely used for evaluating enzymatic activity and inhibition studies, including high throughput screening assays.[53-56]

A fluorescence intensity (FLINT) high-throughput assay to monitor the oxidation of L-DHO to orotate was also recently developed.[57] The assay was originally developed for *Pf*DHODH, but can be applied to other class 2 DHODHs. This assay uses the oxidizing agent resazurin as a second substrate. Resazurin is a redox-sensitive fluorogenic dye that varies from a blue non-fluorescent state to a pink, highly fluorescent state upon reduction to resorufin, monitored at 590 nm (**Figure S1B**, in appendices).[57]

Considering this global introduction regarding DHODHs, we will describe subsequently, divided in chapters, a deeper introduction, methods and the results obtained on the two target enzymes studied in this work, DHODH from *Schistosoma mansoni* (Chapter 2) and DHODH from *Plasmodium falciparum* (Chapter 3).

CHAPTER 2. *SCHISTOSOMA MANSONI* DHODH

2.1 Introduction

Schistosomiasis, is a chronic, debilitating disease caused by blood-dwelling trematodes of the genus *Schistosoma* capable of infecting mammals, birds and reptiles.[58] Five species are capable of infecting humans: *S. mekongi, S. intercalatum, S. mansoni, S. haematobium* and *S. japonicum*. Of these, the last three are more important in public health, being responsible for most cases of schistosomiasis. Only the etiological agent *S. mansoni* is found in Brazil.[59] **Figure 4** illustrates the distribution of these species worldwide.[60] This disease affects over 206 million people (3.3 million DALYs) in more than 70 countries, including sub-Saharan Africa, the Middle East, Southwest Asia and parts of South America.[61, 62] Although this disease is not endemic in other regions, cases have already been reported in Scotland,[63] France, Germany, Italy[64, 65] and China.[66]

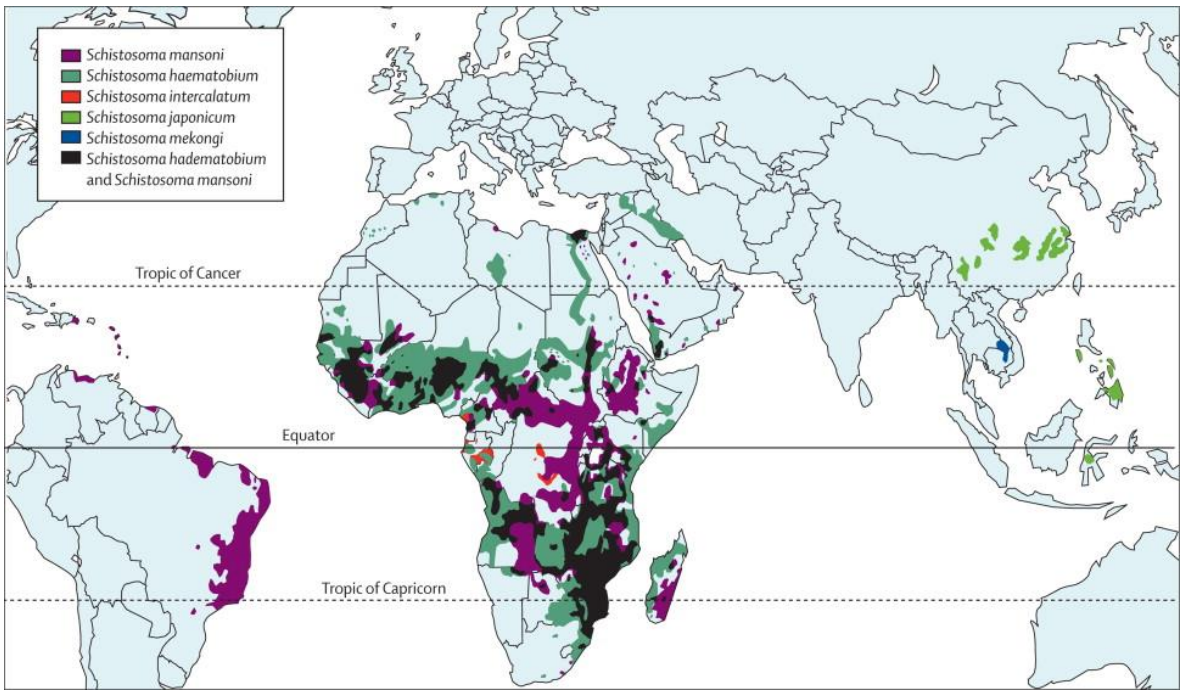

Figure 4. Global distribution of schistosomiasis (extracted from Ferrari and Moreira, 2011 [60]).

The evolutionary cycle of *Schistosoma mansoni* (**Figure 5**)[67] involves an asexual reproduction phase in the intermediate host, a freshwater snail of the genus *Biomphalaria*; a sex-specific phase in the definitive host, the human; and two infective larval stages, cercariae and miracidia, both adapted to favor transmission between hosts.[68, 69]

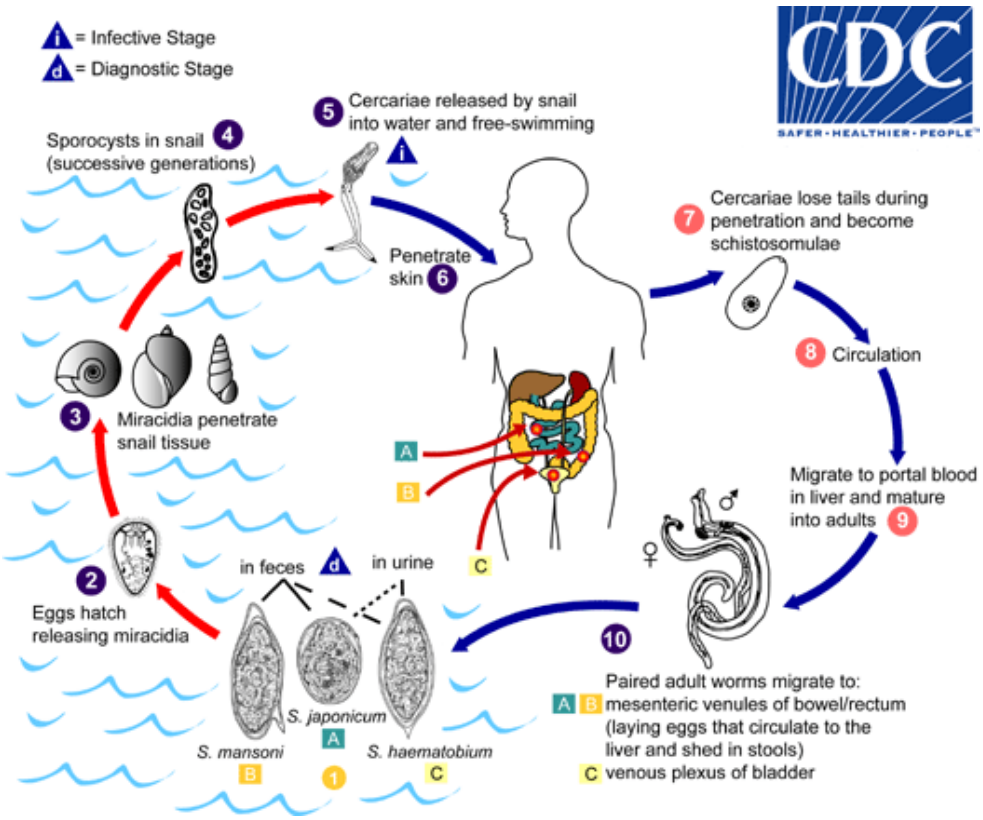

Figure 5. Schistosomiasis parasites life cycle involves two hosts. Eggs are eliminated with feces or urine (1). Under optimal conditions the eggs hatch and release miracidia (2), which swim and penetrate specific snail intermediate hosts (3). The stages in the snail include 2 generations of sporocysts (4) and the production of cercariae (4). Upon release from the snail, the infective cercariae swim, penetrate the skin of the human host (6), and shed their forked tail, becoming schistosomulae (7). The schistosomulae migrate through several tissues and stages to their residence in the veins (8,9). Adult worms in humans reside in the mesenteric venules in various locations, which at times seem to be specific for each species (10). For instance, *S. japonicum* is more frequently found in the superior mesenteric veins draining the small intestine (A), and *S. mansoni* occurs more often in the superior mesenteric veins draining the large intestine (B). However, both species can occupy either location, and they are capable of moving between sites, so it is not possible to state unequivocally that one species only occurs in one location. *S. haematobium* most often occurs in the venous plexus of bladder (C), but it can also be found in the rectal venules. The females deposit eggs in the small venules of the portal and perivesical systems. The eggs are moved progressively toward the lumen of the intestine (*S. mansoni* and *S. japonicum*) and of the bladder and ureters (*S. haematobium*), and are eliminated with feces or urine (1), respectively (extracted from CDC, 2018 [67]).

The parasites have separate sexes and accentuated sexual dimorphism. Adult males measure about 1 centimeter in length and present a foeaceous form, while adult females measure 1.2 to 1.6 centimeters in length, exhibit cylindrical shape, and when they reach one to two years, they can produce up to 400 eggs per day. Infected individuals are

able to eliminate viable *Schistosoma* eggs for 5 years on average. However, some individuals eliminate them for even more than 20 years.[68, 69]

In the chronic phase, the clinical condition of the patient is variable and the disease can evolve into several clinical forms. The intestinal form may be asymptomatic or characterized by diarrhea and abdominal pain. During the hepatointestinal form, there are signs of diarrhea and epigastralgia, with hepatomegaly and characteristic nodules of fibrosis of the hepatic tissue. It is important to mention that the parasite and/or its eggs can still lodge outside the hepatic portal system, generating the ectopic, neurological, vasculopulmonary and renal forms of the disease. Neuroschistosomiasis, for example, is the most severe disabling form of this disease.[60, 69]

The only drug available to treat the patients (Praziquantel) has been on the market for over 50 years and parasite resistance is becoming a significant issue in some areas.[11, 70] Thus, the development of novel drugs to fight schistosomiasis is of utmost importance. One well-established strategy to accomplish this goal is through repurposing of drugs. Herein we propose to take a similar approach by repositioning knowledge and results towards enzymes considered potential drug-targets.

The metabolic pathways responsible for pyrimidine biosynthesis (*de novo* pathway, the salvage pathway[71-73] and thymidylate cycle[74, 75]) are functional in *S. mansoni*. Among the enzymes involved in the *de novo* biosynthesis of pyrimidine nucleotides, *S. mansoni* expresses the flavoenzyme dihydroorotate dehydrogenase (DHODH).[76-78] As described before, this enzyme can be categorized into 2 classes according to their structural features and cellular location.[31] DHODH from humans, *S. mansoni*, *P. falciparum* and *Escherichia coli* pertain to class 2 (**Figure 6**) and, with exception to *Sm*DHODH, they have been extensively studied, and used as a drug target.[41]

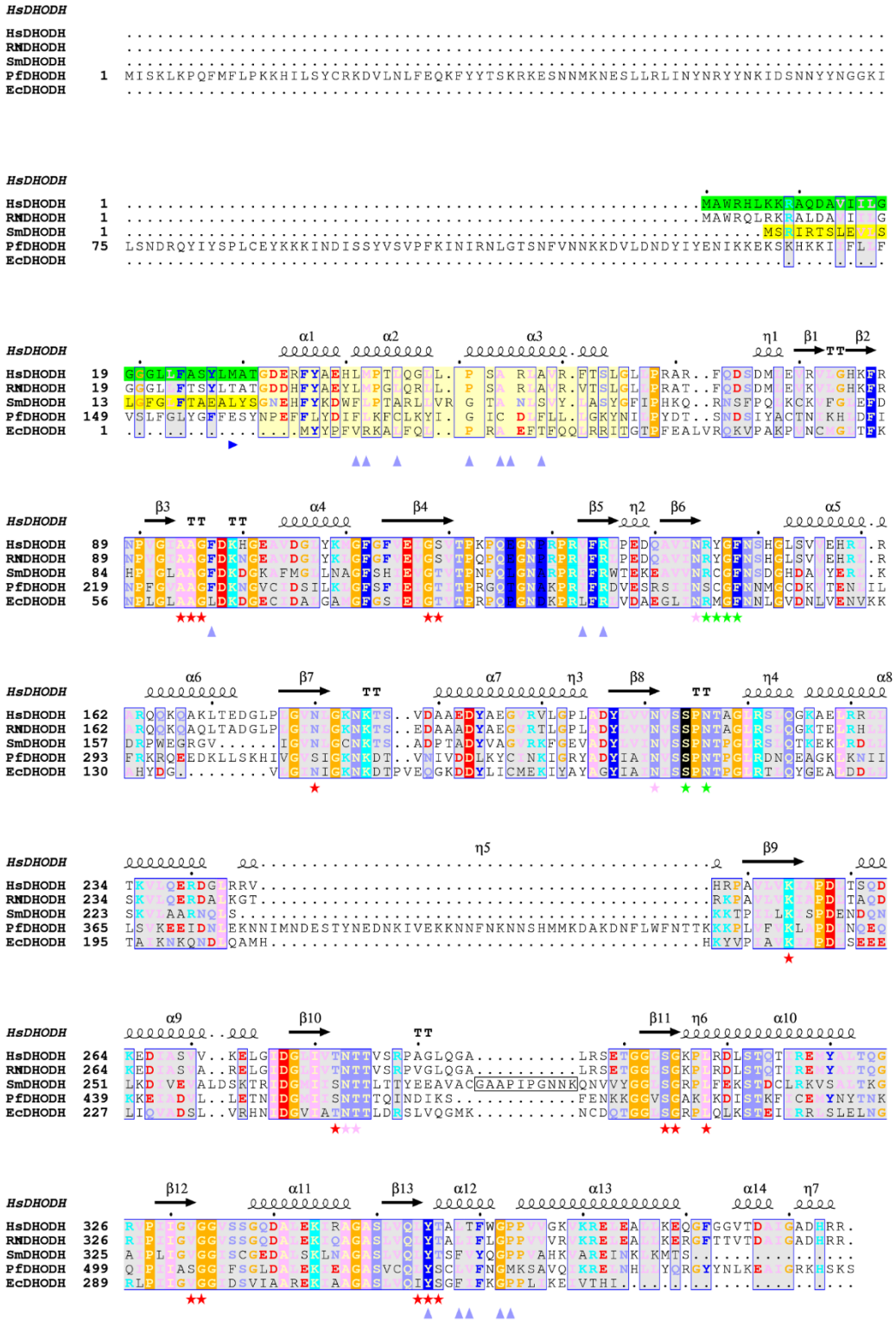

Figure 6. Sequence alignment of selected class 2 DHODHs: *Hs*DHODH (Human DHODH), *Rn*DHODH (*Rattus norvergicus* DHODH), *Sm*DHODH (*Schistosoma mansoni* DHODH), *Pf*DHODH (*Plasmodium falcipurum* DHODH) and *Ec*DHODH (*Escherichia coli* DHODH). Similar residues are colored based on their physical-chemistry properties: polar neutral amino acids (S, T, Q, N) are brown, polar basic residues (K, R,

H) are cyan, polar acidic (D, E) are red, non-polar aromatic (F, Y) are blue, and non-polar aliphatic (A, V, L, I, M) amino acids are pink. G and P are colored in brown. Signal peptide and transmenbrane domain predicted for *Hs*DHODH are highlighted in green. Signal peptide predicted for *Sm*DHODH is highlighted in yellow. Starting point for truncated *Sm*DHODH and *Hs*DHODH constructs is indicated by a blue arrow. N-terminal microdomain responsible to allow protein anchoring to the membrane, harboring the respiratory quinones for FMN reoxidation is highlighted in pale yellow. The serine catalytic residue of class 2 DHODH is highlighted in black. The residues significant for FMN binding, orotate binding, or both FMN and orotate binding are indicated by red, green and pink stars, respectively. Residues recognized as involved in inhibitor binding are indicated by grey arrows. Residue numbering for each sequence is shown at the left. The alignment was performed using MULTALIN[79] and graphically displayed using ESPript 3.0.[80]

Class 2 DHODHs are inhibited by quinone derivatives.[29, 81] Interestingly, chemically similar compounds also display cercaricidal activity:[82] Plumbagin, a naphthoquinone isolated from *Plumbago scandens*;[61] norobtusifolin and kwanzoquinone E, anthraquinones isolated from *Hemerocallis fulva*,[83, 84] resulted in either the mortality or the immobilization of *S. mansoni* cercariae. Derivatives of lapachol and isolapachol have shown activity against different life cycle stages of *S. mansoni*.[85] The relevance of nucleotides production, along with our extensive knowledge regarding class 2 DHODH inhibition by quinone derivatives points out to this enzyme as a druggable target for development of new therapies against schistosomiasis.

The goal of the present project was to perform inhibitory studies against *Sm*DHODH by testing libraries of compounds originally developed as *Pf*DHODH inhibitors. Protein-ligand interaction characterization was initiated by a multi-approach strategy using a combination of structural, biophysical and biochemical techniques.

It is important to mention that previous work has been done regarding *Sm*DHODH and *Hs*DHODH by our laboratory (former master student Juliana S. David).[86, 87] Data previously obtained (initial expression and purification protocols and biochemical characterization of the enzyme) has helped the development of this work. Kinetic parameters, previously determined (**Table 2**) were used to set parameters for activity and inhibitory assays applied and also for the mechanism of inhibition.

Table 2. Kinetic parameters for the steady-state kinetics for the two reactions catalyzed by the enzymes *Sm*DHODH and *Hs*DHODH.

Kinetic Parameters	*Sm*DHODH	*Hs*DHODH
k_{cat} (s^{-1})	31 ± 2	78 ± 4
$K_{M\ DHO}$ (μM)	228 ± 26	286 ± 31
$K_{M\ Q0}$ (μM)	167 ± 21	354 ± 38

Two different constructs were used as models for our studies, named *Sm*DHODH and *Sm*DHODHΔloop. *Sm*DHODH comprises residues Leu[23] to Ser[379], where the predicted mitochondrial targeting peptide and transmembrane region (Met[1] to Ala[22]) has been omitted. *Sm*DHODHΔloop comprises the same fragment as *Sm*DHODH, but a flexible loop (Gly[285] to Lys[294]), not present in other class 2 DHODH, was removed to favor crystallization studies (shown in slate blue in **Figure 7**).

Figure 7 is a homology model built (Modeller)[88] using class 2 DHODH structures previously elucidated from other organisms. Apart from the additional extended loop described before, the figure also shows the α/β barrel catalytic central barrel composed of eight parallel β strands and surrounded by eight α-helices, illustrated in pink, present in both classes 1 and 2. At the top of the barrel, antiparallel β-strands form a domain covering the redox site, likewise at the bottom of the barrel is formed by a pair of antiparallel β-strands. The orotate binding site is located at the top of the barrel, where several strands form the substrate and FMN binding pocket. It is also indicated in the figure, the N-terminal domain in yellow, present only in class 2. The residue highlighted in green is the catalytic residue, Ser[203].

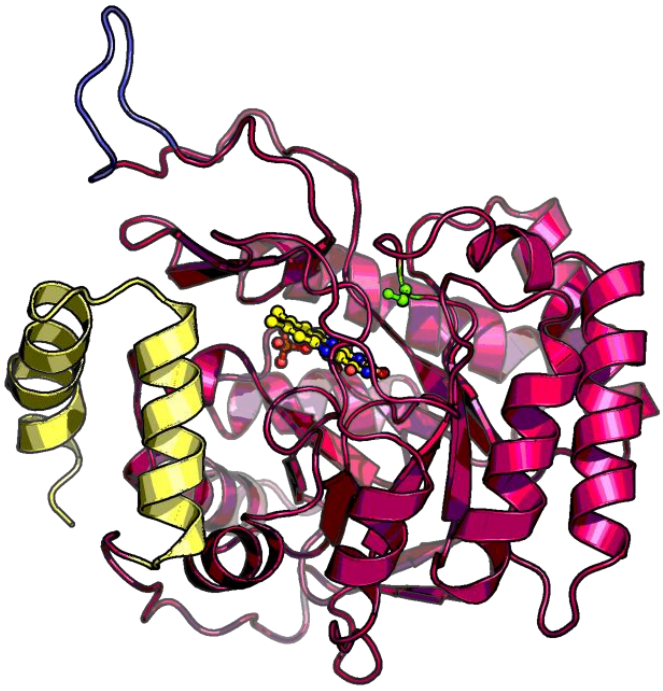

Figure 7. Cartoon representation of *Sm*DHODH homology model.[87] The hydrophobic N-terminal domain composed by two helices is illustrated in yellow. The catalytic central barrel composed of eight parallel β strands and surrounded by eight α-helices is illustrated in pink. The FMN group is located at the top of the barrel and is illustrated as a ball-and-stick model. The catalytic residue Ser^{203} is shown in green. The main structural difference between *Sm*DHODH and other class 2 DHODHs is the presence of a ten-residue peptide that folds as a protuberant subdomain and is illustrated in slate blue.

The studies described next shows the biochemical and biophysical characterization of the enzymes *Sm*DHODH and *Hs*DHODH, as well as the search for ligands, as a first step to evaluate the potential of the selective inhibition of the enzyme *Sm*DHODH. The best inhibitors identified also had their efficacy assessed through *in vitro* assays in the presence of adult *S. mansoni* parasites. The study described in this Chapter can be helpful, in the future, as a therapeutic strategy in the fight against schistosomiasis.

2.5 Conclusions

The present work describes the search for new selective inhibitors against *Sm*DHODH through the use of the strategy called drug repurposing as a strategy to develop new therapeutics to treat schistosomiasis. A total of 185 molecules and analogues that have been develop to treat malaria through the selective inhibition of the enzyme dihydroorotate dehydrogenase were tested and evaluated against *Sm*DHODH. Using both biochemical and biophysical assays with *Sm*DHODH, we were able to identify potent ligands/inhibitors. Several compounds displayed IC_{50} values in the low nanomolar range and some also showed highly selective against the parasite enzyme. Overall, compound 17 (2-hydroxy-3-isopentylnaphthalene-1,4-dione) was considered the best compound for *Sm*DHODH selective inhibition. Inhibition mechanism assays were used to address the understand the binding mode for the best compounds. In order to fully characterize the binding mechanism of those compounds, and to determine the structural basis for enzyme selectivity, structural studies for *Sm*DHODH are currently in progress.

CHAPTER 3. *PLASMODIUM FALCIPARUM* DHODH

3.1 Introduction

According to the WHO, 216 million cases of malaria occurred worldwide in 2016; these led to 445,000 deaths, mainly among children under 5 years of age in African countries. Malaria is found in both tropical and subtropical regions of the planet and in 91 countries (**Figure 8**). The increasing number of malaria cases that have occurred in first-world countries due to globalization have drawn attention to this disease, which, together with AIDS and tuberculosis, represents one of the most serious global public health problems.[105]

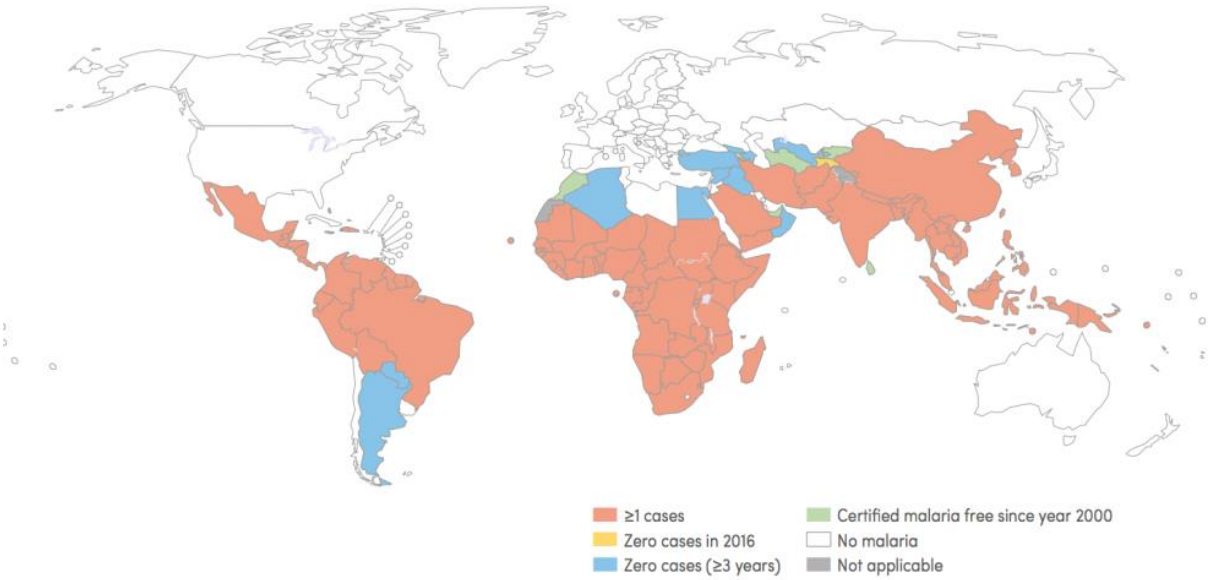

Figure 8. Countries and territories with indigenous cases in 2000 and their status by 2016 (extracted from WHO, 2017 [105]).

Malaria is caused by a protozoan of the genus *Plasmodium*, of which five species are infectious to humans: *Plasmodium falciparum*, which produces the most severe form of malaria; *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium ovale*, which were recently divided into *P. ovale wallikeri* and *P. ovale curtisi*.

These species are primarily transmitted by the bite of infected female mosquitoes of the genus *Anopheles*.[106-108]

Vector control, chemoprophylaxis and chemotherapy with antimalarial drugs are the primary methods used to eliminate or reduce the number of cases of malaria.[109] Most antimalarials operate via mechanisms that target one or two phases of the parasite's life cycle (**Figure 9**).[108] Several drugs are available, each of which acts at a different phase of the parasite's life cycle to prevent development of the parasite in the host.[110] However, the ability of *Plasmodium* species to evade the action of current drugs by developing resistance has become a great challenge to malaria treatment in recent decades, requiring the discovery of more available and effective drugs.[111-113]

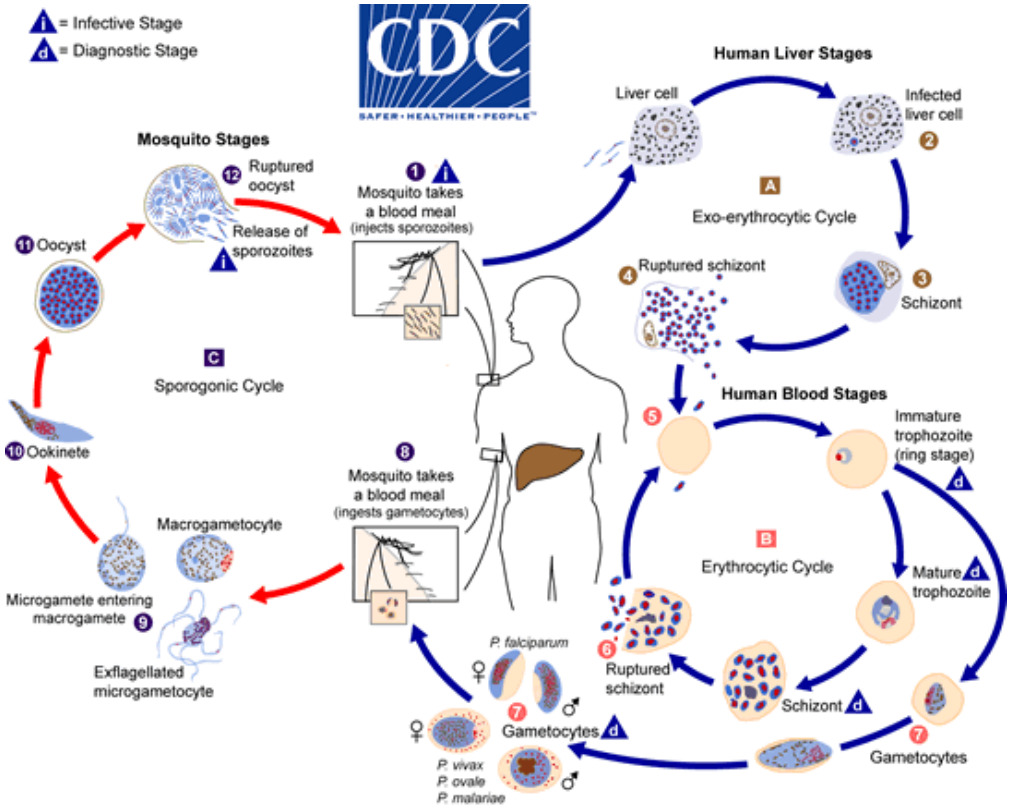

Figure 9. Malaria parasite life cycle involving two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts, which rupture and release merozoites (4). After this initial replication in the liver (exo-erythrocytic schizogony, A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony, B). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (5). Some parasites differentiate into sexual erythrocytic stages

(gametocytes) (7). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites (1) into a new human host perpetuates the malaria life cycle (extracted from CDC, 2018 [108])

The antimalarial drugs that are currently in use fall into the following classes[113-115]:

- Quinoline derivatives. Quinine, an alkaloid isolated from Cinchona bark, was the first compound used to treat malaria. Its use led to the development of synthetic derivatives such as chloroquine (CQ), amodiaquine, primaquine, mefloquine, pyronaridine and piperaquine. Cases of resistance to these drugs have also been reported.[113, 115] The quinolines are active against the erythrocytic forms of *P. falciparum* and *P. vivax*. CQ was originally the most effective drug and has been the first choice for antimalarial treatment for a long time, but abusive use has led to the emergence of CQ-resistant parasites, rendering this drug ineffective in many regions of the world.[115] If used against *P. vivax* and *P. ovale* hypnozoites, primaquine, which inhibits the formation of gametocytes, acts against the slowly developing hepatic forms of *P. vivax* infection that are responsible for relapses.[113]
- Antifolates. These compounds constitute a class of antimalarials that act as schizonticides in the blood and are divided into classes I and II:

Class I. Sulfadoxine, which belongs to the type I class of antifolate drugs, has a structure similar to that of p-aminobenzoic acid. Sulfadoxine interrupts the formation of dihydrofolic acid by inhibiting dihydropteroate synthase, which is necessary for the synthesis of nucleic acids.[116]

Class II. Cycloguanil and pyrimethamine belong to the type II class of antifolate drugs; they inhibit dihydrofolate (DHF) reductase in the parasite, thereby preventing the reduction of DHF to tetrahydrofolate, which is important in the synthesis of nucleic acids and amino acids. DHF reductase inhibitors are potent schizonticidal agents that act on

asexual forms of the parasite. The use of this class of drugs has been reduced due to the capacity of the parasites to develop resistance.[116]

- Artemisinin and its derivatives. Dihydroartemisinin, artemether, arteether and artesunate are known for their ability to rapidly reduce the number of parasites present. These drugs are poorly effective as monotherapies for treatment of malaria due to their low bioavailability and short half-life, and due to cases of resistance, their use is primarily indicated as part of artemisinin-based combination therapy.[105, 117] The endoperoxide bridge of these compounds can undergo reductive cleavage in the presence of ferrous ions from the heme group of hemoglobin, thus generating free radicals that alkylate or modify the proteins of the parasite and lead to its death.[110]
- An antimalarial drug that is used in combination with proguanil for the treatment of malaria is atovaquone. This hydroxyl-1,4-naphthoquinone derivative inhibits oocyst development in the mosquito and pre-erythrocytic development in the liver, interferes with cytochrome electron transport, and also presents low inhibition against the enzyme dihydroorotate dehydrogenase.

Among *P. falciparum* enzymes, *P. falciparum* dihydroorotate dehydrogenase has been identified as an important target in drug discovery. Interference with the activity of this enzyme inhibits *de novo* pyrimidine biosynthesis and consequently prevents malarial infection. *P. falciparum* DHODH (*Pf*DHODH) is a Class 2 DHODH enzyme that contains 569 amino acids (**Figure 6**).

Due to its importance as a drug target, *Pf*DHODH has already been successfully crystallized and had its structure determined 15 times, bound to different ligands. The first *Pf*DHODH structure was determined (2.4 Å) by Hurt et al. in 2006.[118] The *Pf*DHODH crystals complexed with A771726 (teriflunomide) and orotate were obtained by removal of the signal peptide and the transmembrane region and grown using the sitting-drop vapor-diffusion technique at 277 K with sulfate salt as the precipitant, ammonium acetate as a buffer and the detergent pentaethylene glycol monooctyl ether (C8E5) in the crystallization solution. In fact, the use of a detergent in both the purification and crystallization steps is considered obligatory for stabilization of the Class 2 DHODH N-terminal membrane-associated domain.

All of the other DHODHs whose structures were determined later were crystallized using the hanging-drop vapor-diffusion technique at 293 K, also in the presence of sulfate salt and ammonium acetate. The only exception was described by Ross et al. in 2014; those authors used lithium chloride and/or 2-(N-morpholino)ethanesulfonic acid (MES) associated with polyethylene glycol (PEG) 3350.[119] Other components used include PEG 4000, glycerol, dithiothreitol (DTT) and the detergent N,N-dimethyldodecylamine N-oxide, which was used in the purification protocol and/or during crystallization.[19, 21, 37, 53, 119-123]

The first crystal structure of *Pf*DHODH described by Hurt et al. was found to contain a missing or disordered region (residues 375–414) that is not present in Class 2 enzymes such as those of humans or *Schistosoma* species.[118] In fact, removal of a 30-residue-long loop (residues 384–413, shown in **Figure 6**) was found to be necessary to obtain reproducible diffraction-quality crystals.[120] Steady-state kinetic analysis of the construct lacking amino acid residues 384–413 (*Pf*DHODHΔloop) demonstrated that the catalytic efficiency and inhibitor-binding properties of the loop free enzyme were similar to those of the wild-type enzyme.[120] It is worth mentioning that all *Pf*DHODH crystal structures available in the PDB have been solved in the presence of both orotate and potent Class 2 DHODH inhibitors.

The studies described next shows the expression, purification and cloning of the enzymes *Pf*DHODH and *Pf*DHODHΔloop, as well as the search for ligands, as a first step to evaluate the potential of the selective inhibition of the enzyme *Pf*DHODH. Inhibitors identified had their mode of action determined. Crystallization and crystallographic studies were also performed, for the first time in our lab, which can allow the study of binding and design of new candidate molecules. The study described in this Chapter can be helpful, in the future, as a therapeutic strategy in the fight against malaria.

3.5 Conclusions

The increasing number of malaria cases that have occurred in first-world countries due to globalization have drawn attention to this disease. Due to resistance, new drugs are required to overcome this important issue. The present work describes the search for new selective inhibitors against *Pf*DHODH. Using biochemical assays, we were able to identify potent inhibitors. With the interest in identifying the site of inhibition for these compounds, inhibition mechanism assays were performed. Cloning of a new construct, named *Pf*DHODHΔloop, where a protruding loop present in the protein was removed, in order to fully characterize the binding mechanism of the identified inhibitors. Crystals were obtained in different conditions, in which the best data set obtained, was processed at 3.17 Å of resolution. Overall, the structure obtained for *Pf*DHODHΔloop, presents similar folding as to the ones previously solved. Reproducibility was obtained for the crystallization of this enzyme, which guarantees the possibility of acquiring new crystals bound to different compounds.

CHAPTER 4. FINAL REMARKS

4.1 Final Remarks

The work here described and entitled “Repurposing of antimalarial drugs in the treatment of schistosomiasis based on the selective inhibition of the enzyme dihydroorotate dehydrogenase” was carried out between March 2016 and October 2018.

Initially, our work focused on the enzyme dihydroorotate dehydrogenase from *Schistosoma mansoni* (Chapter 2). We were interested in pursuing the search for new potent and selective inhibitors, taking advantage of the previous work performed by the former master student Juliana S. David, which described in her dissertation, the preliminary expression and purification protocols and biochemical characterization for this enzyme. The compounds originally tested had been previously identified as inhibitors of the homologous enzyme from *Plasmodium falciparum*, including molecules under clinical trials such as DSM265 and atovaquone. Potent inhibitors were identified, and the design of analogues (in collaboration with Prof. Flávio Emery) allowed the identification of highly potent (in nanomolar range) and selective inhibitors (cross validation against the human enzyme, *Hs*DHODH).

In order to provide the structural basis for potency and selectivity, different techniques were used, including traditional biochemical assays; inhibition mechanism assays; and the home designed technique ThermoFMN. Considering our lab expertise, structural characterization was also extensively attempted through the use of the X-ray crystallography. Despite testing thousands of conditions, the major challenge of this work was to obtain *Sm*DHODH crystals.

Based on our results, it was possible to characterize three distinct mechanisms of inhibition among the identified ligands: competitive against CoQ_0, non-competitive and mixed-type. Moreover, they effect on thermostability, measured by monitoring the prosthetic group FMN, raised very important questions regarding the mechanism of catalysis adopted by class 2 DHODHs, in particular *Sm*DHODH.

First, by using a small cofactor, we predicted that similar compounds could exploit different interactions in the inhibitor binding site. The competitive ones can reach the end

of the quinone binding site and get in close proximity to the FMN. The non-competitive inhibitors, even though sitting in the same binding pocket, are predicted to bind far away from FMN, holding the helical domain and stabilizing the protein. Mixed-type mechanism, not only corroborate the idea that some of our compounds can exploit different interactions in the pocket, but support previous findings regarding the relevance of the mobility of the helical domain for catalysis as well as binding mechanism of inhibition, including potency and selectivity.

A critical analysis of *Sm*DHODH structure through building a homology model helped us to identify the presence of a flexible loop (Gly^{285} to Lys^{294}), absent in other class 2 DHODHs. Unexpectedly, this region proved to be essential to catalysis and work towards understanding the relevance of this region for protein activity is currently in progress. We recently obtained crystals of our construct *Sm*DHODHΔloop that together with our biophysical assays can provide a starting point to understand the mechanism of catalysis adopted *Sm*DHODH and class 2 DHODH in general. We strongly believe that the understanding on how our target works have a beneficial impact on the rational design of selective inhibitors. Thus, we emphasize here that despite our interest in identifying potent and selective ligands for *Sm*DHODH, our work also focused on contributing for the full characterization of protein function.

In vitro studies using adult *S. mansoni* worms have also been performed. By testing the best inhibitors, it was possible to evaluate their impact on schistosomicidal morphology and activity. Results show a potent activity against the parasites, especially for the compound atovaquone, which it is already a drug in use against malaria. Those exciting, despite preliminary results, provide strong encouragement to keep on pursuing the idea of using inhibition of DHODH, including drug repurposing, as a strategy to search for alternative strategies to treat schistosomiasis.

Considering structural and functional similarity between *Sm*DHODH and *Pf*DHODH, and considering the fact that DHODH is a validated target for malaria, we invested our knowledge on DHODHs and efforts in developing a pipeline to allow the screening and characterization of inhibitors for *Pf*DHODH.

During the development of this work, several molecules have been screened against *Pf*DHODH in our laboratory. This work is performed on regular basis by our Screening Center, under the coordination of Dr. Valquiria Jabor. Several potent inhibitors have been identified from different collaborators in Brazil and abroad. Specifically, for the compounds sent by the Broad Institute of MIT and Harvard in a partnership with MMV (Medicine for Malaria Venture), our laboratory performed not only the determination of inhibitory constants but we were able to map the mechanism of inhibition (as shown in Chapter 3). The protocol developed for this work is now implemented in our pipeline and is being used for other ongoing partnerships. In addition to biochemical studies, the *Pf*DHODHΔloop protein was successfully crystallized and provide us the ability to add structural studies in our pipeline, a required step during drug development based on the selective inhibition of a protein target.

This work is part of a major effort of our laboratory to contribute for the development of new therapeutic strategies to combat neglected diseases as well as the training of human resources in the field of structural biology applied to medicinal chemistry.

4.2 Manuscripts published related to this thesis

Calil, Felipe A.; David, Juliana S.; Chiappetta, Estela R. C.; Fumagalli, Fernando; Mello, Rodrigo B.; Leite, Franco H.; Castilho, Marcelo S.; Emery, Flávio S.; Nonato, M. Cristina. Ligand-Based Design, Synthesis, and Biochemical Evaluation of Potent and Selective Inhibitors of Dihydroorotate Dehydrogenase for the Treatment of Schistosomiasis. *European Journal of Medicinal Chemistry*. v. 106, p. 357-366, 2019.

Nonato, Maria Cristina; De Padua, Ricardo A. P.; David, Juliana S.; Reis, Renata A. G.; Tomaleri, Giovani P.; Pereira, Humberto D.; **Calil, Felipe A.** Structural basis for the design of selective inhibitors for *Schistosoma mansoni* dihydroorotate dehydrogenase. *Biochimie*, v. 158, p. 180-190, 2019.

Hoelz, Lucas V. B.*; **Calil, Felipe A.***; Nonato, Maria Cristina; Pinheiro, Luiz C. S.; Boechat, Nubia. *Plasmodium falciparum* Dihydroorotate Dehydrogenase: a Drug Target Against Malaria. *Future Medicinal Chemistry*, 10(15):1853-1874. 2018 *authors equally contributed to the work.

Reis, Renata Almeida Garcia*; **Calil, Felipe Antunes***; Feliciano, Patricia Rosa*; Pinheiro, Matheus Pinto*; Nonato, Maria Cristina*. The dihydroorotate dehydrogenases: past and present. *Archives of Biochemistry and Biophysics*, v. 632, p. 175-191, 2017. *all authors equally contributed to the work.

Maetani, Micah; Kato, Nobutaka; Jabor, Valquiria A. P.; **Calil, Felipe Antunes**; Nonato, Maria Cristina; Scherer, Christina A.; Schreiber, Stuart L. Discovery of antimalarial azetidine-2-carbonitriles that inhibit *P. falciparum* dihydroorotate dehydrogenase. *ACS Medicinal Chemistry Letters*, v. 8, p. 438-442, 2017.

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