Synthesis of novel *Pf*DHODH inhibitors based on 4-aminocoumarin structures

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Abstract

Malaria is an infectious disease caused by the protozoan of the genus Plasmodium. It is a major problem in third-world countries, with hundreds of millions of infections and millions of fatalities annually. The extreme challenge in malaria management is the resistance of parasites to traditional chemotherapies like chloroquine and artemisinin.

Plasmodium falciparum dihydroorotate dehydrogenase (*Pf*DHODH) was claimed to be one of the hottest malaria drug targets under investigation. *Pf*DHDOH is an enzyme involved in the fourth key step of *de novo* pyrimidine biosynthesis. This way offer potential as targets for drug design, because, unlike the host, the parasite lacks pyrimidine salvage pathway.

In search for new *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH) inhibitors as antimalarial drugs, compounds bearing 5-hydroxy/methoxy-4-aminocoumarin scaffold were developed at Lund University.

The compounds with 5-methoxy-4-aminocoumarin structures were synthetized from resorcinol and should be act as a prodrugs, further test on microsomes are necessary to determinate that. The 5-hydroxy-4-aminocompound was obtained through a melting reaction between 4,5-dihydroxycoumarin and the desired amine.

All compounds will be tested on *Pf*DHODH by Gothenburg University.

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Malaria: the disease

Malaria is a global infectious disease caused by *Plasmodium* parasites. The parasites are transmitted to people through the bites of infected female *Anopheles* mosquitoes, called "malaria vector". Once an infected mosquito bites a human and transmits the parasites, those parasites multiply in the host's liver before infecting and destroying red blood cells (Hoffman & Crutcher, 1996).

Malaria was first identified in 1880 as a disease caused by parasite infection. The name of the disease comes from the Italian word "mal'aria" that means "bad air" (Prevention C. –C., 2015).

In 2015, 95 countries had ongoing malaria transmission. Globally an estimated 3.2 billion people, almost half of the world's population, are at risk of Malaria. WHO estimates 214 million cases of malaria occurred globally in 2015, which the majority of cases (88%) were in African Region, followed by the South-East Asia Region (10%) and Eastern Mediterranean Region (2%), but the number of malaria cases fell from an estimated 262 million in 2000 (Figure 1). Also the number of malaria deaths globally fell from an estimated 839 000 in 2000 to 438 000 in 2015, a decline of 48%. Most of deaths in 2015 were in African Region (90%) followed by South-East Asia Region (7%) and the Eastern Mediterranean Region (2%). About 306 000 malaria deaths were estimated to occur in children under 5 years, almost 70% of the global total. The proportion of children infected with malaria parasites has halved in endemic areas of Africa since 2000, infection prevalence among children aged 2–10 years is estimated to have declined from 33% in 2000 to 16% in 2015. Nevertheless, malaria remains a major killer of children, particularly in sub-Saharan Africa, taking the life of a child every 2 minutes (WHO, World Malaria Report 2015, 2015).

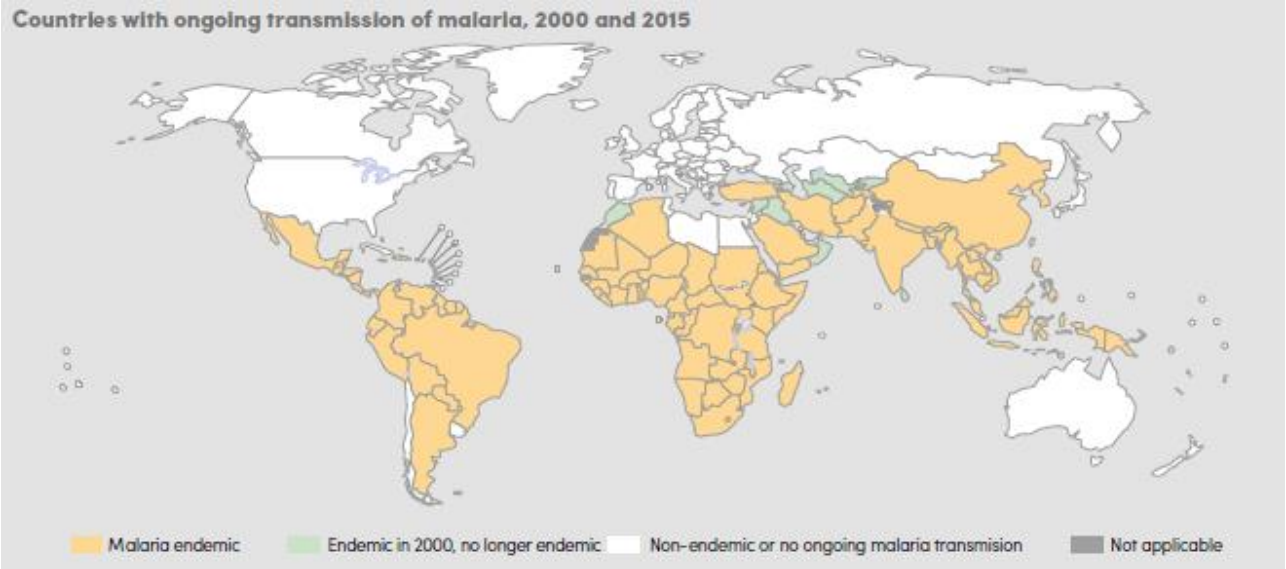


Figure 1. Malaria in the world (WHO, World Malaria Report 2015, 2015).

Principal causes of Malaria

Malaria-infection is caused by infection of red blood cells with protozoan parasite of the genus *Plasmodium* inoculated into the human host (Hoffman & Crutcher, 1996). There are more than 100 types of Plasmodium parasite, but just five are able to infect humans:

- *P. falciparum* it is the most dangerous, located worldwide in tropical and suburban areas, but predominately in Africa, is the cause of 98% of all malaria cases and is responsible for nearly all fatalities in malaria. The strain can multiply rapidly and can adhere to blood vessel walls in the brain, causing cerebral malaria, a complication that can be lethal.
- *P. vivax* –is found mostly in Latin America, Africa, and Asia. Because of the population densities in Asia it is probably the most prevalent human malaria parasite. *P. Vivax* has a dormant liver stage that can activate and invade the blood several months or years after the infecting mosquito bite, causing many patients to relapse.
- *P. Ovale* – which is found mainly in Africa (especially West Africa), it is biologically and morphologically very similar to *P. vivax*. However, differently from *P. vivax*, it can infect individuals who are negative with the Duffy blood group, which is the case for many residents of sub-Saharan Africa.
- *P. malariae* - located worldwide, is the only human malaria parasite to have a three-day cycle. If untreated, *P. malariae* can cause a long-lasting, chronic infection that can last a lifetime.
- *P. knowlesi* - located in Southeast Asia and associated with macaques (a type of monkey). It has a 24 hour replication cycle and can multiply rapidly once a patient is infected, causing an uncomplicated case to severe infection quickly (Prevention C. -C., 2015).

Malaria is spread from one person to another by female mosquitoes (male mosquitoes do not transmit the disease) of the genus Anopheles. There are about 400 different species of Anopheles mosquitoes, but only 30 of these are vectors of major importance. These mosquitoes bite during night-time hours. It has been demonstrated that transmission can occur from transfusion of infected blood or from mother to child in utero, although these instances are very rare when compared with mosquito inoculation. The parasites develop in the gut of the mosquito and are passed in the saliva when an infected mosquitoes bites a person. Uninfected mosquitoes become infected by taking a blood meal from an infected human (Prevention C. -C., 2015).

The principal symptoms of Malaria

Symptoms of Malaria can develop as quickly as seven days after a bite of infected *Anopheles* mosquitoes. The initial symptoms of malaria are nonspecific and are similar to flu-like symptoms and include: headache, lassitude, fatigue, abdominal discomfort and muscle and joint aches, usually followed by fever chills, perspiration, vomiting and worsening malaise. Signs and symptoms in children may be unspecific, leading to delays in diagnosis. Disease progression to severe malaria may take days but sometimes could occur within a few hours. Severe malaria usually manifest with one or more of the following: coma (cerebral malaria), convulsion, hypoglycaemia, acute renal failure, acute pulmonary oedema and sever haemolytic anaemia caused by destruction of red cells. If the infected person is not treated, severe malaria is fatal in the major of cases. The symptoms quickly disappear once the parasites are killed (Flegel, 1976).

Life cycle of parasite

The human malaria parasite has a complex life cycle that requires both a human host and an insect host. In Anopheles mosquitoes, Plasmodium reproduces sexually. In people, the parasite reproduces asexually (by cell division), first in liver cells and then, repeatedly, in red blood cells (RBCs) (Bannister, Lawrence , & Sherman, 2009).

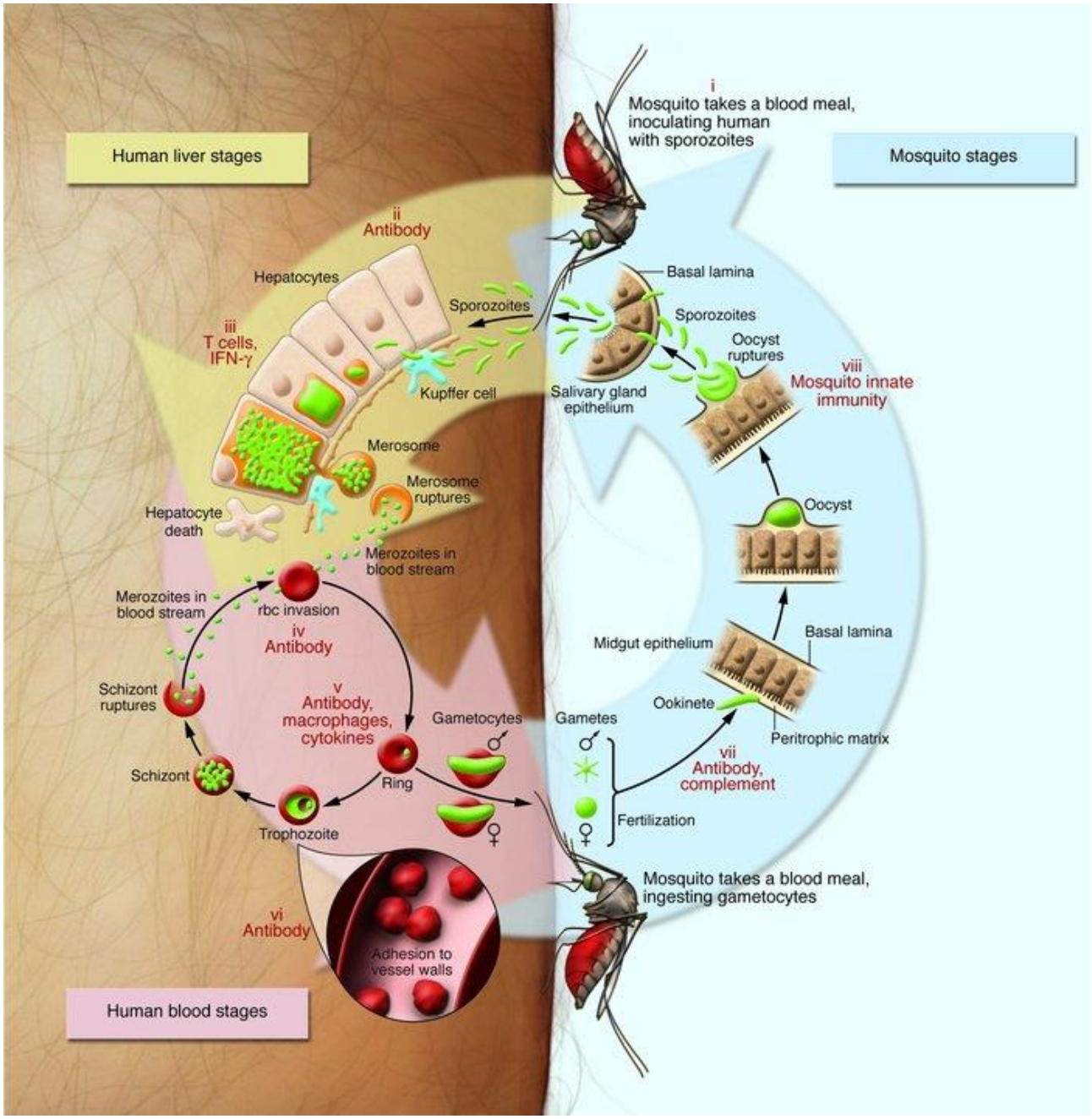


Figure 2. Life cycle of *Plasmodium* (Greenwood, et al., 2008).

Infection of the human host with a *Plasmodium* parasite begins with the bite of an infected Anopheles mosquito it takes in blood. At the same time, it injects saliva that contains the infectious form of parasite, sporozoites, into a person's bloodstream (Figure 2). These motile forms of the parasite rapidly access the blood stream and then the liver, where they invade hepatocytes using a process involving receptor ligand mediated adhesion. The asymptomatic liver stage (known as preerythrocytic stage) of infection lasts a variable period of time dependent to the *Plasmodium* parasite species (5-7 days for P. falciparum, 6-8 days

for P. vivax, 9 days for P. ovale, and 12-16 days for P. malariae). Here, each sporozoite develops into a schizont, a structure that contains thousands of tiny rounder merozoites. When the schizont matures, it ruptures and releases the merozoites into the bloodstream and rapidly invades RBCs.

The blood stages of infection include asexual forms of the parasite that undergo repeated cycles of multiplication as well as male and female sexual forms, called gametocytes, that await ingestion by mosquitoes before developing further. In the asexual erythrocytic cycle, the parasite feeds on the protein portion of hemoglobin and a waste product, hemozoin, accumulates in the host cell cytoplasm (Diseases, 2007). After the parasite undergoes nuclear divisions (it produces more merozoites), the erythrocyte bursts and merozoites, parasite waste, and cell debris are released. The presence of the debris is the cause of the episodes of fever and chills associated with malaria. The merozoites released by the red cell rupture go on to infect more erythrocytes. Time intervals between cell rupture (fever episode), infection of other erythrocytes and then their rupture (new episode of fever) are characteristic of the parasite species.

The asexual stages are pathogenic, and infected individuals can present with diverse sequelae affecting different organ systems. Sexual stage parasites are non-pathogenic but are transmissible to the Anopheles vector, where they recombine during a brief period of diploidy and generate genetically distinct sporozoites. The mosquito becomes infectious to its next blood meal donor approximately two weeks after ingesting gametocytes, a time frame that is influenced by the external temperature.

When a mosquito takes a blood meal from the infected human, the gametocytes begin sexual reproduction in the digestive track of the mosquito and they differentiate in male and female gametes. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle. Each of the developmental stages discussed above represents a potential target at which the life cycle can be interrupted. Vaccines, drugs, and anti-vector measures are being developed to prevent infection, disease, and transmission (Greenwood, et al., 2008)

Strategies for control and elimination of Malaria

WHO recommends a lot of strategies to control and eliminated malaria, which includes:

1. Vector Control
2. Preventive Therapies
3. Diagnostic Testing
4. Treatment With Antimalarial Drugs

Vector control

The most important point of malaria vector control is to reduce human-vector contact and protect individuals from mosquitoes that carry malaria-causing parasites

Preventive therapies

Preventive chemotherapy is the use of complete treatment courses of effective antimalarial drugs for targeted population groups at risk of malaria, with the goal of preventing malaria infection and thereby reducing malaria-related morbidity and mortality.

Diagnostic testing

Malaria is diagnosed by the clinical symptoms and by microscopic examination of the blood. For the biological diagnosis, in 1904, Gustav Giemsa introduced a mixture of methylene blue and eosin stains, microscopic examination of Giemsa-stained blood smears has subsequently become the gold standard of malaria diagnosis (Wongrichanalai, Barus, Muth, Satamihardja, & Wernsdorfer, 2007). Actually all cases of suspect malaria should have a parasitological test (microscopy or Rapid diagnostic test) to confirm the diagnosis. The last one, Rapid Diagnostic Test (RDT) is a device that detects malaria antigen in a small amount of blood, by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip. The result usually a colored test line is obtained in 5-20 min. RDTs require no capital investment or electricity, are simple to perform, and are easy to interpret (WHO, Guidelines for the treatment of malaria, 2015).

Treatment with antimalarial drugs

Antimalarial are designed to prevent or cure malaria. There are two different way to classify the antimalarial. One is according to their action on particular stage of the life-cycle of the parasite, the other is more practical and consider antimalarial drugs by chemical structure since this is associated with important properties of each drug, such as mechanism of action (Bruce-Chwatt, 1962).

Quinine, an aryl amino alcohol, is one of the oldest antimalarial agents and was extracted from cinchona tree bark since 1600s. The cinchona tree produces four alkaloid, these alkaloids are the enantiomeric pair quinine and quinidine and their desmethoxy analogs, chinhonidine (for quinine) and cinchonine (for quinidine) (Song & Eui, 2009). Quinine is lethal for all *Plamodium* schizonts and the gametocites from *P. vivax* and *P.* malariae, but not for *P. falciparum.* The mechanism of action is discussed in the chloroquine section. Quinine has been the main treatment for malaria until the advent of World War II when battle in areas where malaria was endemic led to the search for more effective agents (Gisvold's & Wilson , 2010).

The 4-aminoquinolines are the closest of the antimalarial drugs that are based on the quinine structure. The agent that largely replaced quinine as the antimalarial drug of choice was **chloroquine**. This was

introduced in the 1950s and helped to reduce the malaria spread. However in few years, parasites developed resistant strains to chloroquine and the burden of malaria increased (Patrick, 2013). The principle target of the chloroquine is the heme detoxification pathway (Foye, Lemke, Williams, Roche, & Zito, 2008). In this category of 4-aminoquinolines we find **amodiquine** e **mefloquine** (Gisvold's & Wilson , 2010).

Also 8-aminoquinolines are based on cinchona structures, but they act with a different mechanism. DuringII War World **primaquine** became available this molecule seems to disrupt the parasite's mitochondria. The result is disruption of several processes including maturation into the subsequent forms.

There are two antimalarial drugs that have, in common, the phenanthrene scaffold: Halofantrine and **Lumefantrine**. This drug was developed in China, is very lipophilic and is marketed in combination with the lipophilic artemesinin derived artemether (Gisvold's & Wilson , 2010).

The most important new class of antimalarial agents is the **artemisinins**, which are natural products developed in China beginning in the 1960s. A number of artemisinin derivatives in addition to the parent compound are now available. Although the mechanisms of action of artemisinins are not fully understood, they may include free-radical production in the parasite food vacuole and inhibition of a parasite calcium ATPase. A key advantage of artemisinins is rapid action against all of the erythrocytic stages of the parasite, including transmissible gametocytes, resulting in a rapid clinical benefit and decreased transmission of malaria. (Philip J. Rosenthal, 2008)

Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival. This class of drugs includes effective causal prophylactic and therapeutic agents, some of which act synergistically when used in combination. The drugs used for malaria therapy are divided in two types:

- Type 1 are dihydropteroate synthase enzyme inhibitors (*Pf*DHPS), such as sulfones and sulfonamides whose structures are similar to PABA, with which they compete for the DHPS active site. The main Type 1 is **sulfadoxine**.
- Type 2 antifolates are those that inhibit the parasite DHFR, preventing the DHF reduction to THF described before. These compounds are structurally similar to the pteridine ring of DHF, with which they compete for the active site of DHFR (Delfino, Santos-Filho, & Figueroa-Villar, 2002). **Pyrimethamine**, developed in the 1950s, inhibits the reduction of folic acid and dihydrofolic acid to the active tetrahydrofolate coenzyme form. Proguanil, developed in 1945, is an early example of a prodrug. It is metabolized to cycloguanil, primarily by CYP2C19. The combination of pyrimethamine with sulfadoxine, known as Fansidar™, was introduced in the 1970s after the emergence of parasites resistant to Chloroquine. Fansidar™ blocks the synthesis of dihydrofolate, to block the incorporation of PABA in dihydrofolate, competitively inhibits dihydropteroate synthase, obtaining a sequential block of the synthesis of tetrahydrofolate (Gisvold's & Wilson , 2010).

A synthetic hydroxynaphthoquinone developed in the early 1980s, **atovaquone** has been found to be useful against the *Plasmodium.* It has a highly lipophilic molecule that supposedly interferes with the mitochondrial electron transport and thereby ATP and pyrimidine biosynthesis and in Plasmodia, it is found to target cytochrome bc1 complex and disrupt the membrane potential (Hill & Baggish, 2002).

Also antibiotics as **doxycycline** can be used in the preventive treatment of malaria or in combination with other antimalarial drugs. These agents are active against malaria parasite by inhibiting protein synthesis inside this organelle (Wiesner, Ortmann, Jomaa, & Schlitzer, 2003).

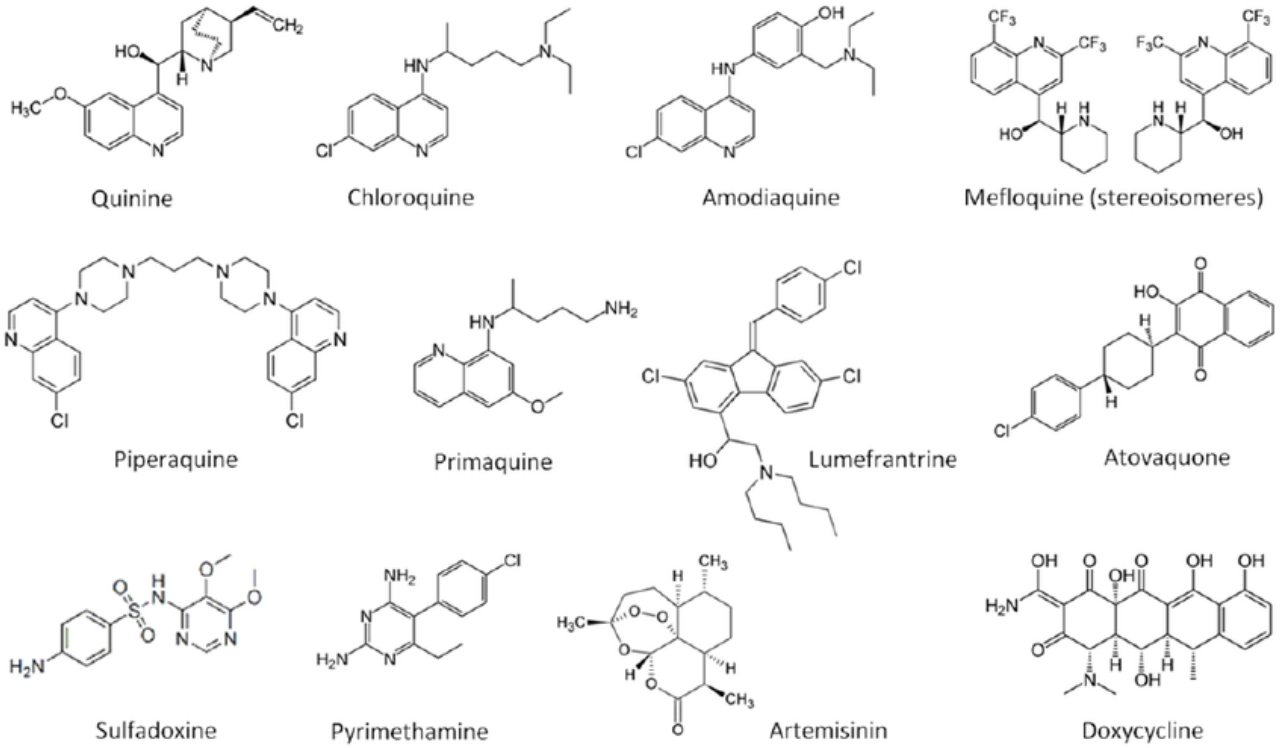


Figure 3. Antimalarial drugs (Makoah & Pradel, Antimalarial drugs resistance in Plasmodium falciparum and the current strategies to overcome them, 2013).

Drug Resistance

The most important issue about the use of antimalarial drugs is the development of resistance. Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher that those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bloland, 2001). It is thought that the development of resistance is due to a spontaneous genetic mutation that confer reduced sensitivity to a given drug or class of drugs. Resistance also develops more quickly where a large population of parasites are exposed to drug pressure (WHO, World Malaria Report 2015, 2015).

Intensive chloroquine chemotherapy has led to the emergence of resistant of *P. falciparum* strains. For chloroquine a different markers have been identified in the past years; however the mechanism by which this happens (resistance) has not been determined. Markers includes *Pf*CRT gene (*P. falciparum* chloroquine resistance transporter) encoding a transmembrane protein of 424 amino acids that is localize in the digestive vacuole membrane and may be involved in drug flux and/or pH regulation. Eight point mutations in *Pf*CRT distinguished chloroquine-resistant from chloroquine-sensitive, in particular K76T mutation confer chloroquine-resistance by exchanging a positively charged residue with an uncharged amino acid, which could allow the active efflux of protonated chloroquine out of digestive vacuole (Plowe, Wellems, & Christopher , Chloroquine-Resistant Malaria, 2001). The *P. falciparum* multidrug resistance transporter 1 (*Pf*MDR1) gene is present on chromosome 5, encodes an ATP-binding cassette (ABC) protein of 1419 amino acid. It has been demonstrated that *Pf*MDR1 reside, like *Pf*CRT, within the membrane of the digestive vacuole. The endogenous function of MDR-like proteins in other organisms consists of the translocation of a variety of substrate including sugars, amino acids, peptides, metals, inorganic ions and toxins. Mutations in MDR transporters lead to decreased intracellular drug accumulation and increased drug efflux. Polymorphisms in *Pf*MDR-1 may also be associated with resistance to mefloquine and artemisinin.

Also the folate pathway is sensible to mutation. Resistance to sulfadoxine-pyrimethamine has emerged in the late 1980s and is now wide-spread with point mutation in both *Pf*DHFR and *Pf*DHPS implicated in resistance. (Petersen, Eastman , & Lanzer, 2001, p. 1551-1562).

Also artemisinin and other derivate are sensible to resistance of *Plasmodium*. When artemisinin and its derivatives were discovered it was thought that this drugs not reported parasite resistance. Instead, after some years, reports from Southeast Asia have described the development of parasite resistance to the drugs. In fact a subset of parasites are cleared from the blood more slowly than previously following ACT treatment, and this phenotype has been currently used to categorise 'artemisinin resistant' parasites. For this reason, to postpone the development of clinically relevant "artemisinin resistance", artemisinin is administered in combination with other drugs and with suitable surveillance methods (White, Dondorp, & Stepniewska , 2009).

Due to the lack of efficient of antimalarial drugs, there is an urgency to identify novel biochemical process of parasite to fill the research pipeline and develop new antimalarial agents. An appealing class of drugs could be dihydroorotate dehydrogenase inhibitors, the enzyme which catalyses one of the main steps in the nucleotides biosynthesis.

Biological background

Purine and pyrimidine nucleotides are major energy carriers, subunits of nucleic acids and precursors for the synthesis of nucleotide cofactor such as NAD and SAM (Ashihara, Hiroshi, & Moffatt, 2002). There are two principal routes for the synthesis of nucleotides: the *de novo* and the salvage pathway. Now we focus our attention on pyrimidine and one enzyme know as DHODH (dihydroorotate dehydrogenase). Since DHODH catalyses one step in the *de novo* biosynthesis of pyrimidine, it is an important enzyme for organisms that need to synthesize their own pyrimidines. Pyrimidines are essential metabolites and are precursors for DNA and RNA biosynthesis. Pyrimidines are available in human cells either by salvage of "used pyrimidines" or by *de novo* synthesis. During rapid cell expansion, the cells can not solely rely on pyrimidine salvage, but a more intensive supply of the pyrimidine through *de novo* synthesis and DHODH function is necessary. Unlike the human host, the Malaria parasite lacks the pyrimidine salvage pathway and has to completely rely on *de novo* biosynthesis of pyrimidine, which makes the parasite vulnerable for inhibition of DHODH function (Reyes, Rathod, Sanchez, Mrema, Rieckmann, & Heidrich, 1982).

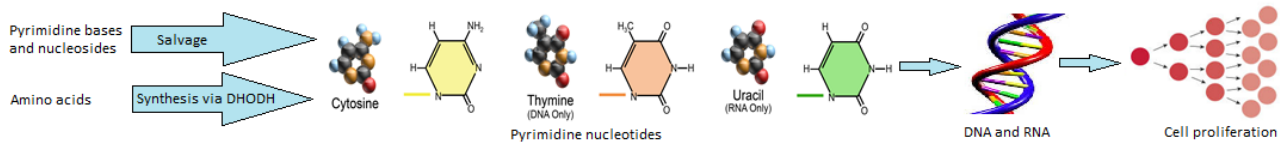


Figure 4. Illustration of the supply of pyrimidines for DNA and RNA synthesis in human cell proliferation.

Unlike *P. falciparum*, humans can salvage preformed pyrimidines as well as synthesize them de novo. Therefore, inhibition of human dihydroorotate dehydrogenase (HsDHODH) is not lethal, but merely slows acquisition of pyrimidines, giving rise to a number of therapeutic responses. Indeed, HsDHODH inhibitors are currently used in the treatment of rheumatoid arthritis and are being evaluated as potential therapeutics for multiple sclerosis, cancer, and even viral infections (Bedingfield, Cowen, Acklam, Cunningham, & Parsons, 2012).

DHODH function

Dihydroorotate Dehydrogenase (DHODH) is an enzyme that catalyses the oxidation of dihydroorotate to orotate. This is the fourth step and the only redox reaction in the *de novo* biosynthesis of UMP, the precursor of all pyrimidine nucleotides. With the exception for DHODH step, all the steps in the pyrimidine biosynthesis are localized in cytosol. The DHODH can be divided into two major classes, 1 and 2, on basis of their amino acid sequences and their cellular location. Members of class 1 includes Gram-positive bacteria, Archea and a few unicellular eukaryotes, they are cytosolic enzyme, while the class 2 enzymes are membrane associated and is found in Gram-negative bacteria and many eukaryotes such as humans, insects, plants and parasites. The two classes are both flavoproteins that use flavin mononucleotide (FMN) as cofactor and electron acceptor. The catalytically important differential between the two classes of enzymes, is the final electron acceptors used by the enzymes used to reoxidize the flavin group and in class 2 a serine replacing cysteine as the catalytic base (Nørager, Jensen, Bjo rnberg, & Larsen, 2002). Class 1 can also be divided into two subclasses of DHODH, one of which uses fumarate as its electron acceptor and other one use NAD+. Class 2 uses coenzyme Q/ubiquinone for their oxidant. DHODH has two redox sites, one where FMN oxidizes dihydroorotate to orotate and another where ubiquinone oxidizes $FMNH_2$ to FMN. The enzymatic oxidation performed by DHODH follow a mechanism described as a two-site ping-pong mechanism, where the electrons from DHP enter FMN from one side and leave FMN at another non-overlapping side that binds the final electron–acceptor (Munier-Lehmann, Vidalain, Tangy, & Janin, On Dihydroorotate Dehydrogenases and Their Inhibitors and Uses, 2013).

DHODH structure

The major difference between the class 1 and class 2 is that class 1 DHODHs is soluble (cytosolic enzymes), because they lack about 30 residues that mediate mitochondrial targeting and insertion into the membrane.

The membrane-association motif is adjacent to a pair of α-helices, α1 and α2, that are also specific of type 2 DHODH. These two helices are connected by a short loop and form the small N-terminal domain. The slot between α1 and α2 form a hydrophobic funnel where ubiquinone is probably inserted and connects at its extremity with the FMN binding cavity. This small N-terminal is directly connected to the larger C-terminal domain of the human enzyme where binding sites for the substrate and the FMN are located. Again, this large domain folds into an α/β-barrel with a central structure of eight parallel β strands surrounded by eight α helices (Munier-Lehmann, Vidalain, Tangy, & Janin, On Dihydroorotate Dehydrogenases and Their Inhibitors and Uses, 2013). A tunnel is formed between the N-terminal helices and α/β-barrel domain that almost consist of hydrophobic amino acids. The tunnel begins at the surface of the protein and ends adjacent to FMN and is the site where DHODH inhibitors bind (Walse, et al., 2008).

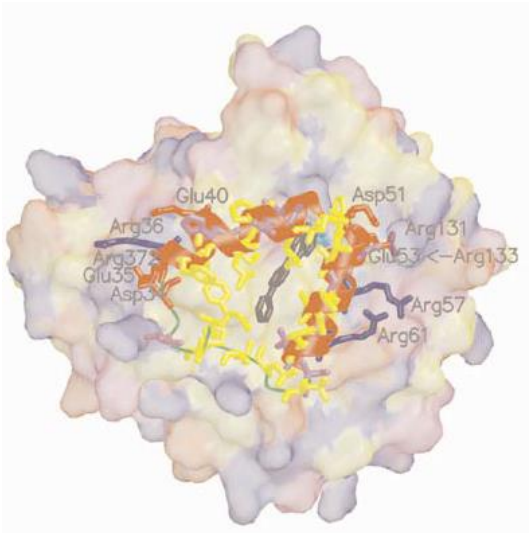


Figure 5. Surface of DHODH (Liu, Neidhardt, Grossman, Ocain, & Clardy, 2000).

The inhibitor binding site was originally thought to bind a cofactor ubiquinone during the hand-over of electron from FMN. The structure of human DHODH in complex with an antiproliferative (Figure 6) agent was solved in 2000 and shows that the site for ubiquinone and inhibitor do not overlap, but nevertheless the binding of an inhibitor prevent the binding of the ubiquinone.

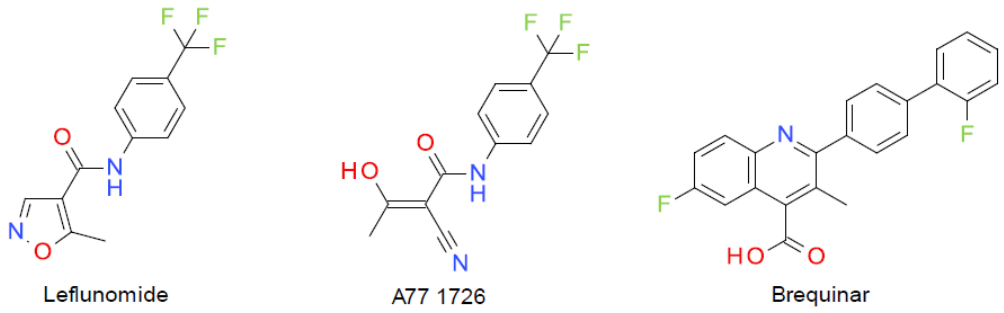


Figure 6. Antiproliferative agents active on DHODH (Boa, Canavan, Hirst, Ramsey, Stead, & McConkey, 2005).

High-resolution structures of human DHODH in complex with a brequinar and the active metabolite of leflunomide (A771726) have been solved. Both, the brequinar and A771726 bind in the same region: in the tunnel formed by the N-terminal extension that folds into a helical domain, near the flavin mononucleotide. Metabolic studies have indicated that lefunomide (used for autoimmune diseases) is rapidly processed in vivo to a primary metabolite A771726, which mediate immunosuppressive effects. A771726 binds to the same site as brequinar. The carbonyl is hydrogen bonded to a water, which in turn is bound to Arg136, while the enolic OH is hydrogen bonded to Tyr356. The trifluoromethyl-containing aromatic ring makes numerous hydrophobic contacts with residues in the tunnel. A crucial specificity determinant between family 1 and 2 DHODHs is the hydrophobic channel in which the inhibitors are bound in human (Walse, et al., 2008).

The first analysis of *Pf*DHODH was reported in 2005, containing the ligand A771726. The structural basis for the observed species selectivity is evident through comparison of the X-ray structures of the human and malarial enzymes, which show that the A77 1726 binding site is highly variable in amino acid sequence between the enzymes from the two species. While both human and malarial structures contain A77 1726 in this site, but A77 1726 is a poor inhibitor of the malarial enzyme. The *Pf*DHODH ligand binding pocket is 55 Å, while the dimensions in human and rats are larger (715 and 760 Å, respectively) than the previous one. The smaller size of the *Pf*DHODH tunnel explains why larger inhibitors as A771726, brequinar and atovaquone, are ineffective against *Pf*DHODH (Hurt, Widom, & Clardy, 2006).

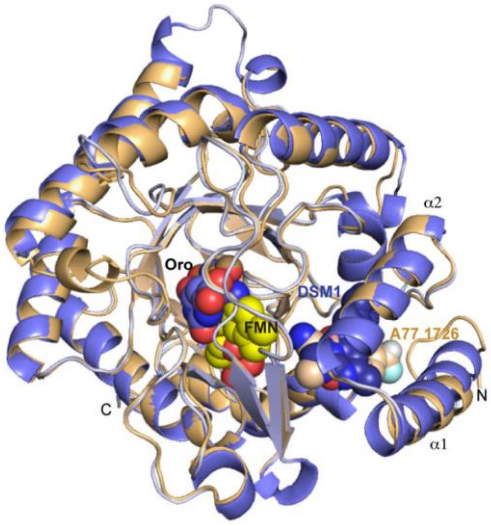


Figure 7. X-ray structure of *Pf*DHODH (Phillips & Rathod, 2010).

The *Pf*DHODH inhibitor-binding has two components: the hydrogen-bond site between Arg256 and His185 (and nearest to FMN) and the adjacent hydrophobic pocket that is lined with residue in part contributed by helices 1 and 2. The amino acids residues nearest FMN show the least conformational flexibility and generally align closely between all known structures, whereas the residues in the hydrophobic pocket have been observed in multiple conformations. Recent studies of the X-ray structure of *Pf*DHODH with a selective N-phenylbenzamide inhibitor reveals novel binding site including the stacking interaction with Glu181/Glu182 and the 'halogen bond' are novel interactions that have not been observed in other *Pf*DHODH–inhibitor complexes, and both are likely to contribute significant binding energy to the interaction. These data show that the flexibility of the *Pf*DHODH inhibitor-binding site allows it to form tight interactions with diverse structural classes of small molecules, enhancing its value as a drug target (Deng, Matthews, Rathod, & Phillips, 2015).

Target diseases of DHODH and current treatment

Human dihydroorotate dehydrogenase (DHODH) represents an important target for the treatment of hyperproliferative and inflammatory diseases. In the cell DHODH catalyses the rate-limiting step of the de novo pyrimidine biosynthesis. DHODH inhibition results in beneficial immunosuppressant and antiproliferative effects in diseases such as rheumatoid arthritis (Baumgartner, et al., 2006). A DHODH inhibitor, lefunomide, is used as the immunosuppressive ingredient in the marketed drug Avara®. 1998 Avara was approved for treatment of rheumatoid arthritis in adults. Brequinar, another DHODH inhibitor, was discovered for its anticancer effects. Unfortunately this highly potent human DHODH inhibitor has been found to suffer from limited therapeutic window (Boa, Canavan, Hirst, Ramsey, Stead, & McConkey, 2005). Vidofludimus (SC12267) is a novel oral immunomodulator inhibiting dihydroorotate dehydrogenase (DHODH) and the expression of pro-inflammatory cytokines and interferon-gamma (Herrlinger, et al., 2013). Also DHODH of *P. falciparum* is a relevant antimalarial target.

*Pf*DHODH inhibitors

The *Pf*DHODH inhibitors are competitive with CoQ10 and inhibit the reoxidation of $FMNH_2$. Site-directed mutagenesis data supported a model whereby the CoQ-binding site does not overlap with the inhibitor site, but inhibitors could block electron transfer between FMN and CoQ10 or they could stabilize a conformation that exclude CoQ-binding. The first example of DHODH inhibitor with a nanomolar activity in malaria whole cell assays, with triazolopyrimidine structure was named **DSM1**. This compound is a potent inhibitor of the enzyme but not metabolically stable. The second compound**, DSM2**, shows the same behaviour of its analogue DSM1. The replacement of the naphthalene portion of DSM1 with a p-trifluoromethylphenyl substituent led to compound called **DSM74**, which was more metabolically stable in human liver microsomes in vitro but less potent in malaria whole cell assays than DSM1 (Deng, et al., 2009)**.** All of these three bind in the same site.

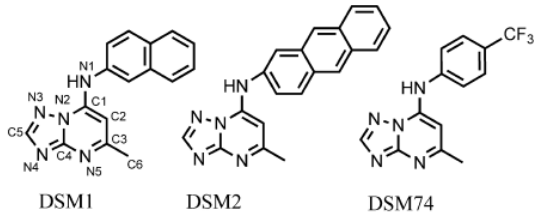


Figure 8. Structure of triazolopyrimidine (Deng, et al., 2009).

How DMS structures are bound to two sub-sites is shown in Figure 9. The triazolopyrimidines form two hydrogen bonds to the protein, the nitrogen in naphtylamine position of DSM1 interacts with histidine 185 and the pyridine nitrogen interact with arginine 265. The other pocket is completely hydrophobic and binds naphthyl (DSM1), anthracenyl (DSM2) and phenyl-trifluoromethyl (DSM74) groups.

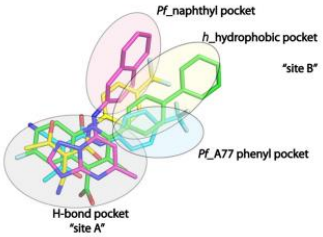
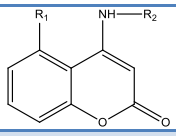
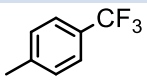
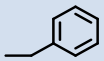
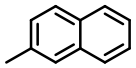
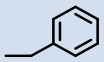
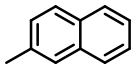


Figure 9. Structure of *Pf*DHODH-DSM1 (pink) (Deng, et al., 2009).

In the search of new *Pf*DHODH inhibitors, several methods can be used. One is to use human DHODH inhibitors as starting points and re-design them into *Pf*DHODH. From previous studies (Fritzson, et al., 2010) we know that scaffold of 4-hydroxycoumarin (or 4-hydroxy-2H-chromen-2-one), was a good starting point. They were projected as inhibitors of *h*DHODH. In this case two part of the coumarin could interact with arginine 136; either the 4-hydroxy group or the lactone part of the pyran ring. Furthermore, an aromatic rings have been introduced in 3 position that pointing towards the hydrophobic site. It was also observed that 4,5-hydroxycoumarins are more potent and higher ligand efficiencies than the corresponding 4-hydroxycoumarin, which suggests that is participate in a polar interaction.

The analysis of crystal complex *Pf*DHODH-triazolopyrimidines was used to identify limitations and interaction possibilities in the binding site. There is a small difference in direction of the hydrophobic subsite in *Pf*DHODH and *h*DHODH. The change in direction makes expansion at the coumarin 4-position more suitable for malaria inhibitors instead of the 3-position that was beneficial for the human coumarin inhibitors. Inspiration from the binding mode of triazolopyrimidines led to introduce aromatic amines in 4-position of the coumarin scaffold. Recent studies made by Fritzson and co-workers show that the 4-amino-5-hydroxycoumarins are selective against *Pf*DHODH and below is shown the compounds with best activity against *Pf*DHODH.



Comp.	R_1	R_2	IC_{50} (µM)$^{\alpha}$ *Pf*DHODH	IC_{50} (µM)$^{\alpha}$ *h*DHODH
76	H		1.7±0.13	>100
77	H		12±2.5	>100
78	H		0.78±0.038	>100
81	OH		9.2±1.0	100
82	OH		0.26±0.0118	57±1.6

As reported in the above table, two of the 4-amino-substituted coumarins (**78** and **82**) were found to have submicromolar activity in the *Pf*DHODH assay and are both N-substituted with the naphtyl moiety. Also compound **76**, which is N-substituted with 4-trifluoromethyl-phenyl group, is close to the submicromolar breakpoint. If we compare compound **78** with **82** and **77** with **81**, there is a benefit to substitute the coumarin with a hydroxyl group in 5-position. It is possible that the hydroxyl group interact with tyrosine 528, however the interaction seem not to be optimal since the difference is less than a factor of three. The structure-activity relationship for the coumarins is similar to the SAR of the published triazolopyrimidine. Fritzon and co-workers did an X-ray analysis of compound **81** in complex with *Pf*DHODH, which confirmed a proposed binding mode of the 4-amino-5-hydroxycoumarins (unpublished, Figure 10).

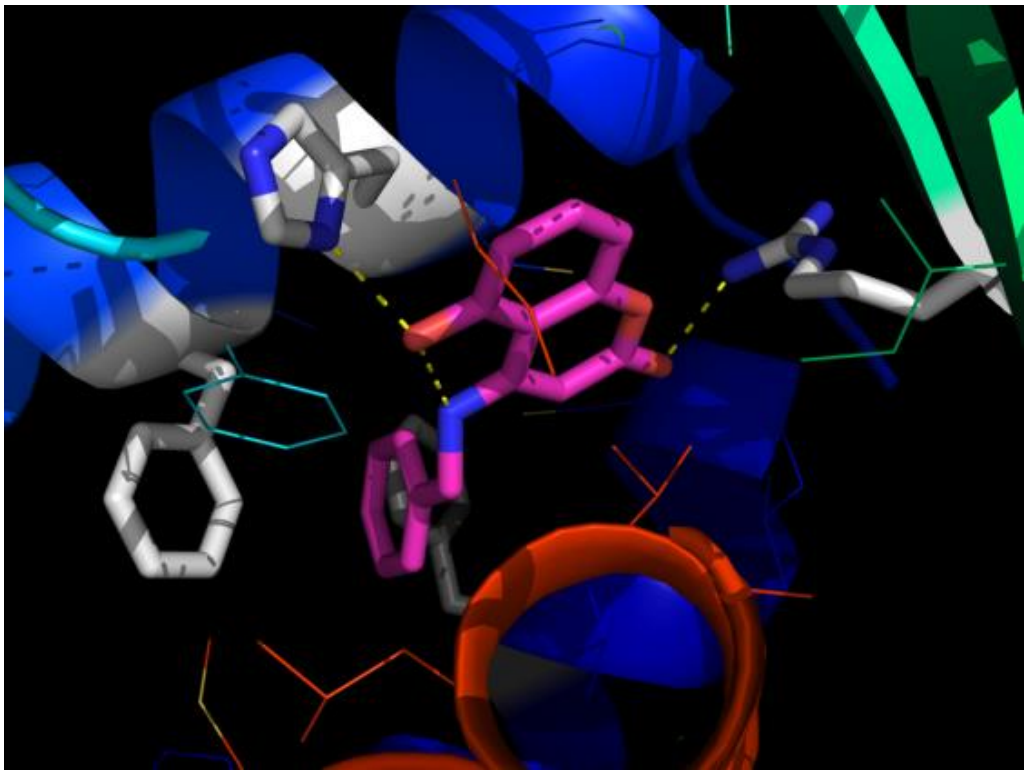


Figure 10. X-ray analysis of compound 81 in complex with *Pf*DHODH (unpublished).

Coumarin uses the lactone (oxygen of the ring and the carbonyl oxygen) for interaction with arginine 265; the amine acts as a hydrogen bond donor to both the internal 5-hydroxy group and histidine 185. The 5-hydroxy donates its hydrogen to histidine 185. The distance between the 5-hydroxy group and tyrosine 528 is less than 4.5 Å, the possibility of hydrogen bond formation between them (mediated by water molecule) should not be neglected. The benzyl group gives π-stacking interactions with phenylamine 227 and 188 in the hydrophbic site.

Aim of thesis

The objective of this research project was to identify new 4,5-dihydroxycoumarin derivatives and to make structural modification in order to improve the interaction between the ligand and enzyme.

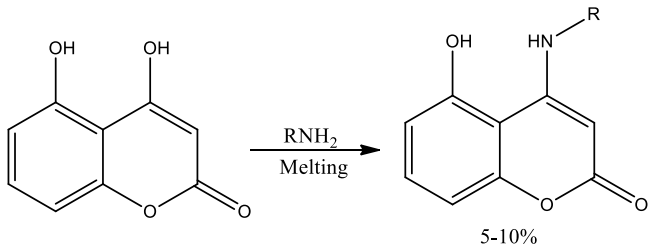
From the X-ray analysis and inhibition data of previous we know what is important:

- Lactone structure
- OH free in 5’ position
- Amino group in 4’ position
- Hydrophobic substituent on the amine

We aimed at synthetizing compounds with these features. Furthermore, we wanted to improve the selectivity inhibition of the *Plasmodium falciparum* dihydroorotate dehydrogenase (DHODH) for potential treatment of Malaria.

Synthesis

In the first part of our research project, we investigated if it was a possibility to avoid the melting reaction, by which earlier 4-amino-5-hydroxycoumarins compounds were obtained (Scheme 1) between 4,5-dihydroxycoumarin) and a desired amine. The problem of this reaction was the low yield of the product.



Scheme 1. Melting reaction between 4,5-dihydroxycoumarin and amines.

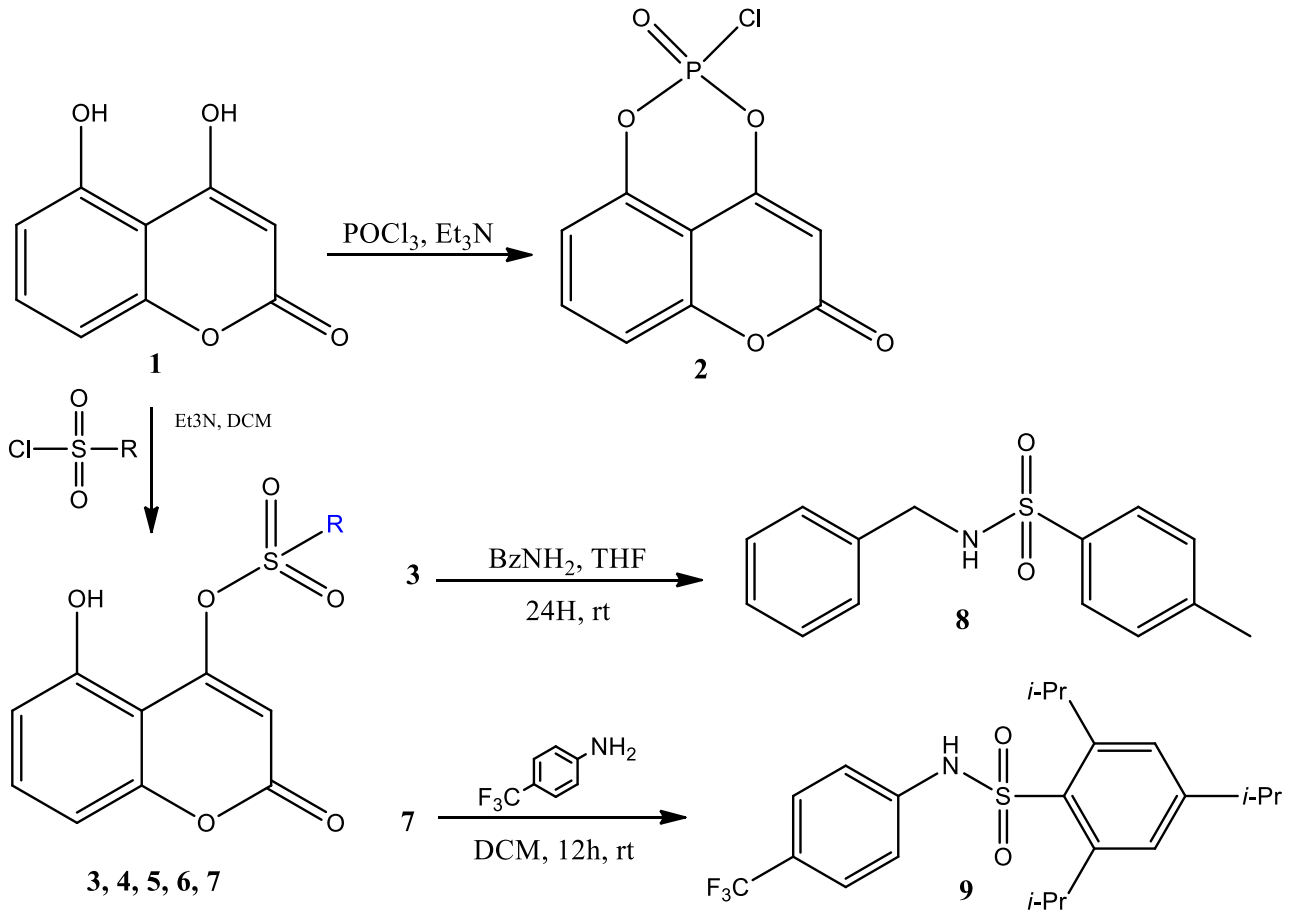
To reach 4-amino-5-hydroxycoumarins, the hydroxyl group in 4' position was replaced with a better leaving group (Scheme 2).

The first attempt was to substitute with chloride, a reaction reported in the literature to work with 4-hydroxycoumarin. However, with 4,5-dihydroxy-2H-benzopyran-2-one [**1**] and the reagent, phosphoroxy chloride, a cyclization occurred over 4-hydrox and 5-hydroxy to give [**2**]. Several attempts were tried with different conditions, but all produced compound [**2**]. Treatment with trifluoromethansulfonic anhydride, also failed probably because triflate products were unstable.

Then the attention was focused on aryl sulfonates as leaving groups. With all of them we obtained a mixture with two products: the proper one, with sulfonyl group in the 4-position and unwanted one where the substitution is occurred in the 5-position. All sulfonylation reactions were conducted with a solution of 4,5-dihydroxy-2H-benzopyran-2-one in DCM with 1 equivalent of base (usually Et_3N, but also pyridine and isopropylamine) and 1 equivalent of sulfonyl chloride compound. Two products then were separated with flash chromatography (DCM-MeOH 20/1). The best yield was obtained with tosylate [**3**], but unfortunately [**3**] reacted with benzylamine to give the corresponding sulphonamide [**8**].

In a last attempt 2,4,6-triisopropylbenzenesulfonyloxy chloride [**7**], was used to obtain a more hindered sulfonate avoid the formation of the corresponding sulphonamide, however also in this case a sulphonamide [**9**] was formed from [**7**] and trifluoromethylaniline.

In conclusion, all attempts to replace the hydroxyl group with a leaving group that could be substitute with primary amine failed.



Sulfonyl R groups

3 **56%**

p-Toluenesulfonyl chloride (TsCl)

4 **23%**

4-Nitrobenzenesulfonyl chloride (NsCl)

5 **32%**

3,5-Bis(fluoro)benzenesulfonyl chloride

6 **28%**

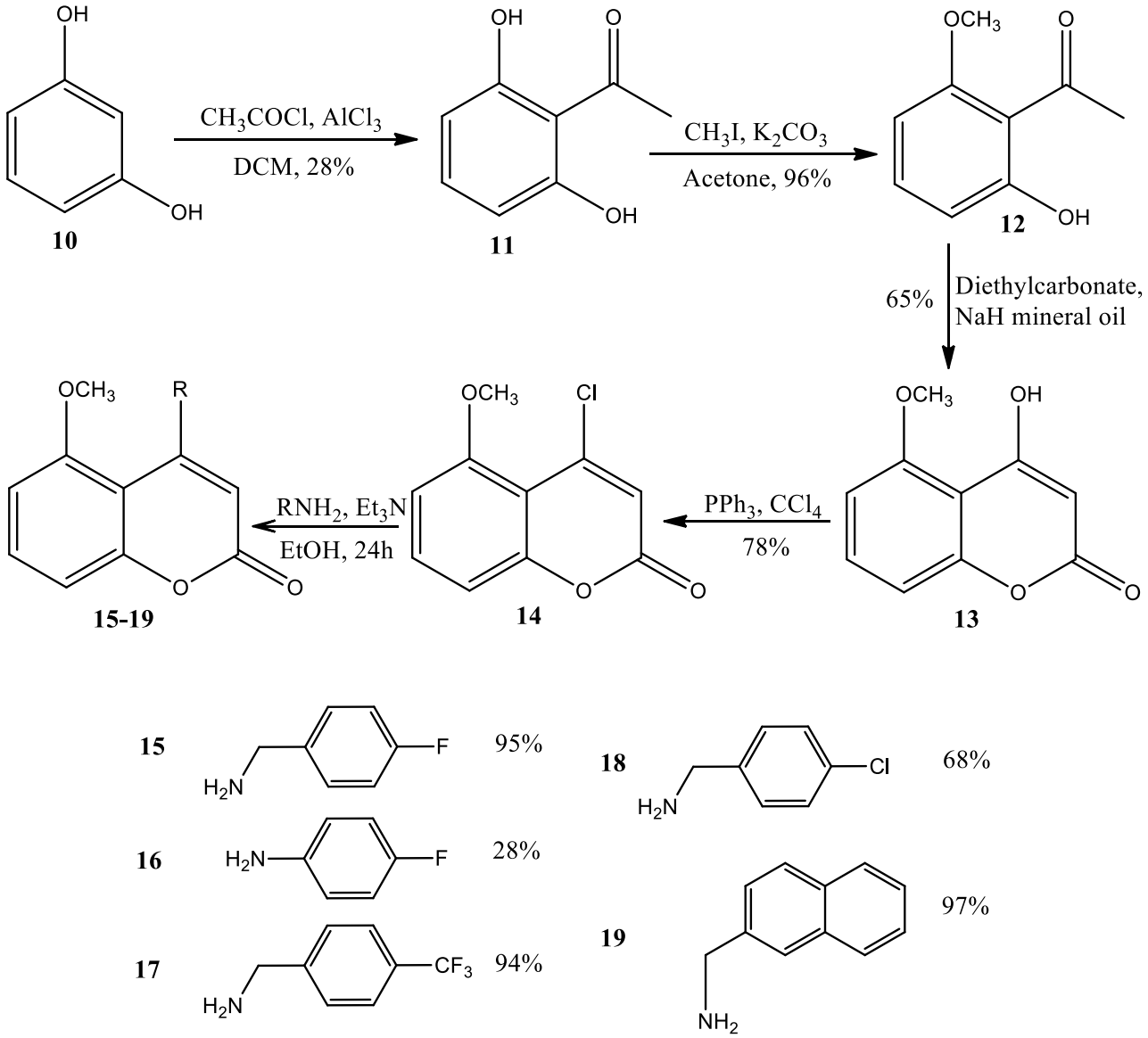
3,5-Bis(trifluoromethyl) benzenesulfonyl chloride

7 **41%**

2,4,6-Triisopropylbenzenesulfonyl chloride

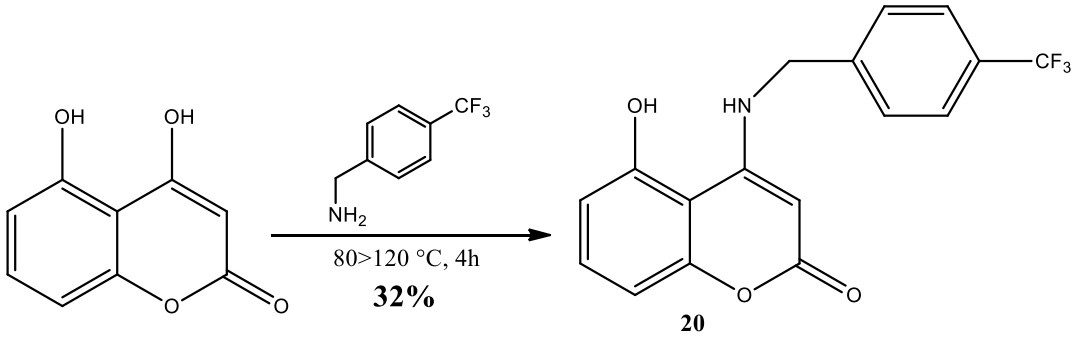
Scheme 2. Attempts to convert the coumarin 4-OH to leaving groups

The second part of our research project was focused to find a new synthetic route towards 4-amino-5-hydroxycoumarins (Scheme 3). Starting with an acylation of Resorcinol [**10**] with acetyl chloride gave 2',6'-dihydroxyacetophenone. This reaction was not improved mainly because 2',6'-dihydroxyacetophenone is commercially available. In the second step monomethylation of [**11**] with methyl iodide to give 2-hydroxy-6-methoxyacetophenone [**12**] followed by reaction with sodium hydride in diethyl carbonate gave 4-hydroxy-5-methoxycoumarin [**13**]. Now it was possible replaced the hydroxyl group with a chloride, carbon tetrachloride/triphenylphosphine was used and the 4-chloro-5-methoxycoumarine was obtained [**14**]. All the steps then were improved with a larger amount of starting material, in a classic scaling-up. In the last step, the chloro derivate reacted with the proper amine in ethanol and give our compounds in high yield with nucleophilic amines. The less reactive 4-fluoroaniline gave as expected a lower yield. Unfortunately the last step, deprotection of the methoxy group did not work. We used different reagents such as chlorotrimethylsilane and boron tribromide in different conditions like dry ice/acetone bath at -78 °C, ice at 0 °C and room temperature but all attempts were without success.



Scheme 3. Synthesis of 4-arylamine-5-methoxycoumarins

In the last scheme is shown the melting reaction of the 4,5-dihyxroxycoumarin [**1**] with an aromatic amine (Scheme 4). This reaction was done to obtain a 4-amino-5-hydroxycoumarin compound for comparing amine substitution efficiency with the method in Scheme 3. If compared with other melting reaction, it worked with a relatively yield. Moreover, we could also directly compare *Pf*DHODH inhibition properties of the product of this melting reaction [**20**] with the methoxy derivative [**17**] synthetized in Scheme 3.



Scheme 4. Melting reaction to give 5-Hydroxy-4-[(4-trifluoromethyl)benzyl]amino-2H-benzopyran-2-one [20].

Conclusion

The project aimed at synthetizing new compounds as selective inhibitors of *Pf*DHODH. Unfortunately, we had a lot of problems with the substitution of 5-hydroxycoumarin with sulfonate leaving groups in position 4. However, 5-methoxy derivatives were possible to chlorinate and substitute with primary amines at position 4. Hence, an important result of the project is that we developed a method to introduce 4-arylamino groups with yields far superior to the direct melting reaction with 4,5-dihydroxycoumarin. Furthermore, the 5- methoxy compounds are possible pro-drugs, because enzymes maybe are able to cleave the methoxyl group into a hydroxyl group. Hence, the 5-methoxy-4-aminocoumarins should be tested in e.g. microsome assays.

All the final compounds are currently tested for *Pf*DHODH inhibition at Gothenburg University.

Future Perspectives

If 4-amino-5-methoxycoumarin compounds will be active *in vitro*, will be possible synthetize new coumarins with the pathway showed above (Scheme 3). Moreover, it is interest to replace the methoxy group with a different group that could be cleaves easily in the last step (e.g. benzyloxy group).

Materials and Methods

Starting materials and reagents were obtained from commercial suppliers. NMR spectra were recorded on a Bruker Avance II 400 MHz spectrometer at ambient temperature. ^{1}H-NMR and ^{13}C-NMR spectra were assigned using 2D-methods (COSY and HMQC). Chemical shifts (δ), determined from residual solvent peaks, are reported in ppm relative to TMS while Js are in Hz. Reaction were monitored by TLC using alumina-backed silica gel plates (Merck 60F254) and visualized using UV light. HRMS was determined by direct infusion on a Waters XEVO-G2 QTOF mass spectrometer using electrospray ionization (ESI). All final compounds had a purity ≥95% (254 nm) recorded on a Hewlett Packard series 1100 HPLC, column Waters Acquity CSH C18, 1.7 um, 2.1 x100 mm.

Chemical procedure

2',6'-Dihydroxy-acetophenone [11]

A 50 mL flame-dried round-bottom flask equipped with a stir bar was charged with resorcinol (0.5 g, 4.541 mmol) and $AlCl_3$ (1.211 g, 9.08 mmol). Methylene chloride (5 mL) was added and the reaction vessel was capped with a rubber septum attached to a balloon of nitrogen and submerged in an ice bath. Once chilled, the acetyl chloride (0.3244 mL, 4.541 mmol) was added dropwise over 1-2 minutes. The syringe was rinsed with CH_2Cl_2 (1 mL) and the reaction was left to slowly warm to room temperature overnight. After the allotted reaction time, CH_2Cl_2 was removed with a steady stream of nitrogen. Crushed ice was then added until the reaction was quenched (caution: addition of water is exothermic). The quenched reaction mixture was then transferred to a separatory funnel with EtOAc (40 mL) and was extracted with 1M HCl (3x15 mL) followed by brine (1x30 mL). The organic layer was died over $MgSO_4$ and evaporated. The crude product was either subjected to silica gel chromatography using a gradient of Heptane/EtOAc (starting from 5:1) as solvent to give the desired product as a yellow solid (0.1983 g, 28% yield); ^{1}H-NMR(DMSO-d^{6}): 11.81 (s, 2H); 7.23 (t, 1H); 6.36 (d, 2H); 2.63 (s, 3H). ^{13}C-NMR(DMSO- d^{6}): 215.5, 170.7, 162.2, 136.5, 110.8, 107.5 (2C), 33.6.

2-Hydroxy-6-methoxyacetophenone [12]

Potassium carbonate (2.755 g, 1 mol) and methyl iodide (1.255 mL, 1 mol) were added in 12 mL of acetone to solution of 2',6'-dihydroxyacetophenone (3 g, 1 mol). The solution was refluxed for 8 hours. After this time the solvent was removed under reduced pressure. The resulted residue was dissolved in water (50 mL) and extracted with DCM (3*30 mL). The combined organic layer was washed with brine and dried over Na_2SO_4. Mixture was filtrated and concentrated under vacuum to obtain our compound like a waxy yellow solid (3.127 g, 95%); ^{1}H-NMR($CDCl_3$): 13.26 (s, 1H); 7.34 (t, 1H); 6.56 (d, 1H); 6.39 (d, 1H); 3.90 (s, 3H); 2.67 (s, 3H). ^{13}C-NMR($CDCl_3$): 205.3, 164.6, 161.6, 136.1, 111.3, 110.7, 101.1, 55.6, 33.5.

4-Hydroxy-6-methyl-2H-benzopyran-2-one [13]

A solution of 2-hydroxy-6methoxacetophenone (3.0521 g 18.37 mmol) in diethyl carbonate (18 mL) was added dropwise to NaH (60% dispersion in mineral oil) (3.674 g, 91.85 mmol) at 0 °C. The resulting mixture was heated at 100 °C for 3 h when it was cooled first to room temperature and then to 0 °C. Water was then carefully added dropwise to quench residual NaH. The mixture was extracted into diethyl ether. The aqueous phase was acidified to pH 3 with HCl. The resulting precipitate solid was collected by filtration, washed sequentially with water followed by heptane and then dried in the oven at 90 °C overnight. A colourless solid was obtained (2.267 g, 65%). ^{1}H-NMR($CDCl_3$): 9.53 (s, 1H), 7.48 (t, 1H), 7.02 (d, 1H), 6.79 (d, 1H), 5.68 (s, 1H), 4.07 (s, 3H). ^{13}C-NMR($CDCl_3$): 166.0, 162.8, 156.2, 155.1, 132.5, 111.4, 105.5, 104.9, 93.0, 57.1.

4-Chloro-5-methoxy-2H-benzopyran-2-one [14]

A mixture of 4-hydroxy-5-methoxy-2H-benzopyran-2-one (2.267 g, 11.78 mmol) and triphenylphosphine (4.3313 g, 16.51 mmol) in carbon tetrachloride (16 cm^3) was heated at reflux for 6 h, cooled and left to stir overnight at room temperature. The mixture was diluted with methylene chloride (50 cm^3), washed with water (3 x 30 cm) and dried with Na_2SO_4. The crude obtained after the removal of thesolvent was either subjected to silica gel chromatography using a gradient of Heptane/EtOAc (starting from 4:1) as solvent to give the desired product (1.961 g, 79%). ^{1}H-NMR($CDCl_3$): 7.49 (t, 1H), 6.97 (d, 1H), 6.79 (d, 1H), 6.46 (s, 1H), 3.93 (s, 3H). ^{13}C-NMR($CDCl_3$): 158.8, 157.5, 154.8, 148.6, 133.17, 115.7, 109.8, 108.2, 107.2, 56.3

General procedure for amine derivatives

To a solution of 4-Chloro-5-methoxy-2H-benzopyran-2-one (250 mg, 1 equivalent) in 20 mL of ethanol, Et3N (1 equivalent) and the corresponding amine (1 equivalent) were added and mixture was refluxed for 24 hours. The solution was allowed to cool, and the solid was filtered off and washed with a small portion of ethanol giving the compounds.

4-(Fluoro)benzylamine-5-methoxy-2H-benzopyran-2-one [15]

Yield: 95%. ^{1}H-NMR($CDCl_3$): 7.93 (bs, 1H), 7.44 (t, 1H), 7.35 (d, 2H), 7.10 (d, 2H), 7.01 (d, 1H), 6.76 (d. 1H), 5.15 (s, 1H), 4.41 (d, 2H), 3.95 (s, 3H). ^{13}C-NMR($CDCl_3$): 163.6, 162.7, 157.3, 155.5, 155.2, 132.3, 131.2, 129.0, 128.9, 116.1, 115.8, 111.5, 105.6, 104.8, 82.6, 56.5, 46.5. ESI-MS $[M+H]^+$: 300.1036. Purity: 98.14%

5-Methoxy-4-[(trifluoromethyl)phenyl]amino-2H-benzopyran-2-one [16]

Yield: 28%. ^{1}H-NMR($CDCl_3$): 9.23 (bs, 1H), 7.68 (d, 2H), 7.48 (t, 1H), 7.42 (d, 2H), 5.66 (s, 1H), 4.06 (s, 1H). ^{13}C-NMR($CDCl_3$): 162.3, 154.3, 153.2, 153.1, 143.5, 133.5, 127.5 (2C), 125.8, 125.1, 124.8, 124.6, 123.9, 118.0, 115.5, 84.6, 56.6. ESI-MS $[M+H]^+$: 336.0848. Purity: 97.43%

5-Methoxy-4-[(trifluoromethyl)benzyl]amino-2H-benzopyran-2-one [17]

Yield: 94%. ^{1}H-NMR($CDCl_3$): 8.02 (bs, 1H), 7.64 (d, 2H), 7.44 (m, 2H), 7.43 (t, 1H), 6.98 (d, 1H), 6.76 (d, 1H), 5.10 (s, 1H), 4.50 (d, 2H), 3.97 (s, 3H). ^{13}C-NMR($CDCl_3$): 162.3, 157.3, 155.5, 155.3, 140.6, 131.7, 130.1 (2C), 127.3, 126.0 (2C), 111.5, 105.6, 104.8, 83.1, 56.6, 46.7. ESI-MS $[M+H]^+$: 350.1004. Purity 98.01%

4-(Chloro)benzylamine-5-methoxy-2H-benzopyran-2-one [18]

Yield: 68%. ^{1}H-NMR($CDCl_3$): 7.93 (bs, 1H), 7.40 (t, 1H), 7.36 (m, 2H), 7.28 (m, 2H), 6.99 (d, 1H), 6.74 (d, 1H), 5.15 (s, 1H), 4.39 (d, 2H), 3.94 (s, 3H). ^{13}C-NMR($CDCl_3$): 162.6, 157.3, 155.5, 155.2, 135.0, 133.7, 131.7, 129.2 (2C), 128.6 (2C), 112.5, 105.6, 104.6, 82.8, 56.6, 46.6. ESI-MS $[M+H]^+$: 316.0751. Purity 98.61%

5-Methoxy-4-[(naphtalen-1-ylmethyl)-amino]-2H-benzopyran-2-one [19]

Yield: 97%. ^{1}H-NMR($CDCl_3$): 8.10 (bs, 1H), 7.86 (m, 4H), 7.43 (m, 3H), 7.42 (t, 1H), 6.99 (d, 1H), 6.74 (d, 1H), 5.35 (s, 1H), 4.57 (d, 1H), 3.99 (s, 3H). ^{13}C-NMR($CDCl_3$): 171.2, 162.8, 157.3, 155.6, 155.4, 133.9, 133.4, 133.0, 131.5, 129.0, 127.8, 127.7, 126.6 (2C), 126.2, 125.1, 111.4, 105.5, 82.6, 56.5, 47.5. ESI-MS $[M+H]^+$: 332.1289. Purity: 97.78%

5-Hydroxy-4-[(4-trifluoromethyl)benzyl]amino-2H-benzopyran-2-one [20]

4,5-dihydroxy-2H-benzopyran-2-one (1 equiv) was heated together with 4-(trifluoromethyl)benzylamine (1 equiv) up to 90 °C where the material melts together for 3 hours. The crude product was mixed with hot EtOAc and allowed to cool. The solid was filtered off and washed with a small portion of ethanol giving the compound. Yield: 32%. ^{1}H-NMR(DMSO-d^{6}): 10.06 (bs, 1H), 7.73 (m, 3H), 7.63 (d, 1H), 7.57 (d, 2H), 7.16 (t, 1H), 6.52 (d, 2H), 6.44 (d, 2H), 4.73 (s, 1H), 4.59 (s, 2H), 4.05 (s, 1H). ^{13}C-NMR(DMSO- d^{6}): 162.1, 157.6, 155.7, 143.5, 132.0, 129.5, 128.2 (2C), 126.1, 125.9, 125.8, 125.7, 112.0, 104.7, 103.7, 79.3, 45.5. . ESI-MS $[M+H]^{+}$: 336.0848. Purity: 97.90%

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