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Thioredoxin Reductase As A Potential Target For Novel Antimalarial Drug Development

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Abstract:
The human malaria parasite Plasmodium falciparum possess an increasing threat to human health in the tropicalregions of the world, and the validation and assessmentof possible drug targets is required for the development of new antimalarials. The erythrocytic stages of the parasite responsible for the pathology of the disease in humans, are under enhanced oxidative stress and are particularly vulnerable to exogenous challenges by reactive oxygen species. The disruption of the antioxidant or redox systems of the parasite is a feasible way to interfere with their development during erythrocytic schizogony; targeting these antioxidant or redox system of the parasite is the major property of a potent antimalarial drug .Over the last decades, malaria parasites have been rapidly developing resistance against antimalarial drugs, which underlines the need for novel drug targets. The pressing need for new antimalarials has led to an increase in research focused on the Plasmodium parasites causing malaria. To cope up with the oxidative stress to which they are exposed during the erythrocytic stages of their life cycle the parasite exhibit a crucial antioxidant system comprised of Thioredoxin reductase (TrxR) which is an enzyme required to maintain redox equilibrium in Plasmodium species, and is thus a promising target for new antimalarials. TrxR of Plasmodium falciparum is a central focus of this paper since it is the only Plasmodium TrxR that has been crystallized and P. falciparum is the species that causes most malaria cases. P. falciparum TrxR is indeed targeted in the parasite by specific inhibitors with antimalarial activity. Pre-screening and identification of antimalarial compounds from the Malaria Box by Medicines for Malaria Venture (MMV) have also revealed about five such compounds showing significant PfTrxR inhibitory activity. Based on efficacy of the antimalarial and sensitivity of the drug animal models are also employed to test PfTrxR inhibitors. Finding promising molecules with new mechanisms of action to catalyse the drug development in order to combat antimalarial resistance is the present need of the hour.

Keywords:*Plasmodium falciparum thioredoxin reductase; malaria; antimalarial; screening for mechanism of action; Malaria Box*

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I. Introduction:

Malaria, one of the most threatening infectious diseases is characterized by high fevers and chills, is caused by *Plasmodium sp.*, a protozoan parasite that are transmitted to humans via a bite from an infected Anopheles mosquito. The malarial parasite critically depends on its intracellular redox balance in the Anopheles vector and the human host. *P. falciparum* relies on two antioxidant systems to relieve oxidative stress and maintain redox equilibrium within the parasite (Jortzik *et al*., 2012). These are the glutathione and thioredoxin systems. The thioredoxin system consists of thioredoxin reductase (TrxR), its substrate, thioredoxin (*Pf*Trx1), and NADPH. *Pf*TrxR is essential for the intraerythrocytic stages of the human pathogen *P. falciparum* (Krnajski Z et al., 2002) and has been validated as a drug target.

TrxR contains a dithiol or disulfide active site and serves as a cellular protein disulfide reductase, while Trx is an electron donor for a multitude of enzymes, including ribonucleotide reductases, thioredoxin peroxidises and methionine sulfoxide reductases (Holmgren *et al*., 2000). *Pf*Trx1 is an active electron donor for vital protein targets including ribonucleotide reductase and a whole set of peroxiredoxins (Jortzik E, Becker K, 2012). The functions of Trx directly depend on its reduction by TrxR. *P. falciparum* expresses a cytosolic and a mitochondrial high molecular weight TrxR from one gene by alternative translation initiation (Kehr S et al., 2010). *Pf*TrxR belongs to a family of homodimeric pyridine nucleotide- disulphide reductases. This flavoenzyme thioredoxin reductase (TrxR) thus became a focus of attention of drug discovery approaches.

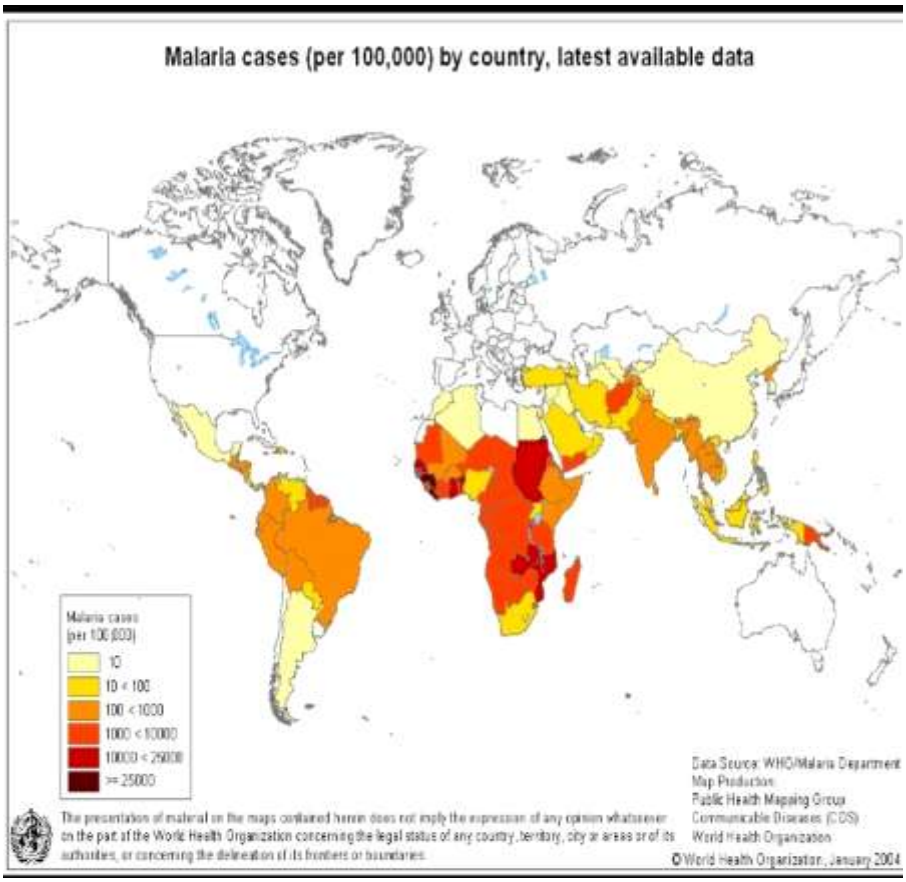
The *Plasmodium* parasite is equipped with a complex Trx system based on three Trxs located in the cytosol, the parasitophorous vacuole, and the endoplasmic reticulum (kehr S et al., 2010). The function of Trx directly depend on its reduction by TrxR. The TrxR of *Plasmodium falciparum* is of high molecular weight between 55 and 60 kDa (Coombs et al., 2000) with significant differences between the active sites of parasite and host proteins (Williams C H et al., 2000). TrxR is homodimeric, NADPH- dependent disulphide oxidoreductase that possess one FAD cofactor bound per subunit and residues from both subunits contribute to

the two active sites per homodimer (Gilberger et al., 1997; Krnajski et al., 2000). The C- terminal redox- active centre of the *Plasmodium* TrxR contains a specific CysXXXXCys motif (Gilberger et al., 1998; Wang et al., 1999; Tamura and Stadtman, 2002). Importantly, the P. falciparum TrxR gene has been genetically validated as a potential drug target as its disruption proved to be lethal for the parasites (Krnajski et al., 2002). Thus, the protein provides good potential for the design of inhibitors that specifically target parasite survival.

This review presents an overview of the life cycle of the malarial parasite and the mechanism of how it invades the host cell, the classic symptoms of the malaria disease, the diversity and evolutionary significance of the enzyme thioredoxin reductase and also why the enzyme thioredoxin reductase(*Pf*TrxR) act as a potential drug target for the antimalarials. Moreover, the future prospects for studies on potentiality of TrxR as target for antimalarial drugs against *Plasmodium* species which is infectious to humans would also pave way for the development of more specific inhibitors of *Pf*TrxR as potential new antimalarial drug leads.

II. Epidemiology of the disease : Malaria

Malaria is one of the most important public health problem in term of morbidity and mortality, causing more than 200 million cases and 655.000 deaths every year (World Malaria Report, 2011). According to the World Health Organization (WHO) Malaria Report 2011, a total of 106 countries in the world are at risk of transmission of malaria infection. A total of 216 million estimated malaria cases occurred in 2010, 81% of which were reported in the African Region, followed by South East Asia (13%) and Eastern Mediterranean Region (5%). The total number of malaria deaths was estimated to be 655.000 in 2010; 91% of whom occurred in the African Region, 6% in South-East Asia and 3% in Eastern Mediterranean Region. The absolute number of people at risk for malaria infection increased from 0.8 billion in 1900 to 3.3 billion in 2010, as a consequence of the absolute increase of the population living in malaria-endemic regions. However, between 2005 and 2010 malaria cases decreased from 244 million to 216 million; moreover, malaria mortality rates showed a global reduction of 26% between 2000 and 2010. India however contributes about 77% of the total malaria in South East Asia. The world Health Organization estimates 300-500 million malaria cases annually. In 2018, an estimated 228 million cases of malaria worldwide is been reported and the estimated number of deaths is about 405000 in 2018. The WHO African Region carries a disproportionately high share of the global malaria burden. In 2018, about 93% of malaria cases and 94% of malaria deaths is been reported from this region.



(Source: WHO Malaria Department; Map Production: Public Health Mapping Group; Communicable Diseases (CDS); World Health Organization, January 2004)

Life cycle of the malaria causing parasite**:** It involves two different hosts: the definitive host or vector, which is the mosquito of the genus Anopheles, followed by a vertebrate intermediate host, such as a human. Haploid sporozoites present in salivary glands of female mosquitoes are injected into the vertebrate host during a blood meal. Sporozoites are inoculated into the blood stream of humans with the bite of the female infected *Anopheles* (Figure 1) released from their salivary glands through the proboscis. These sporozoites then enter hepatocytes and transforms into a trophozoite. It is the exoerythrocytic stage.In *P. vivax* and *P. ovale*, they remain in a quiescent stage, known as hypnozoites. The trophozoites undergo several rounds of mitosis and meiosis to form schizonts which consist of several daughter merozoites. The erythrocytes are lysed due to overcrowding by the merozoites, which are then released. The merozoites then infect new erythrocytes and repeat the blood schizogony.The parasite forms a parasitophorous vacuole, to allow for its development inside the erythrocyte. Each synchronous cycle of schizogony in *P. falciparum* is of 48 hours duration which coincides with the symptomatic peaks of fever. After several such cycles, some merozoites develop into gametocytes. They are of two types – male (microgametocytes) and female (macrogametocytes). It is the sexual stage of malaria and is infective to mosquitoes. They are again taken up by the mosquito during a blood meal.

Parasites undergo sporogonic cycle in the mosquito. Inside the stomach, the microgamete penetrates the macrogamete, producing a zygote. The zygote becomes elongated and motile (ookinete) and invades the wall of the midgut and develops into oocyst. It grows and ruptures to release sporozoites, which migrate to the salivary glands between the 10th and the 18th day, and are injected into humans during a blood meal. This completes their life cycle.

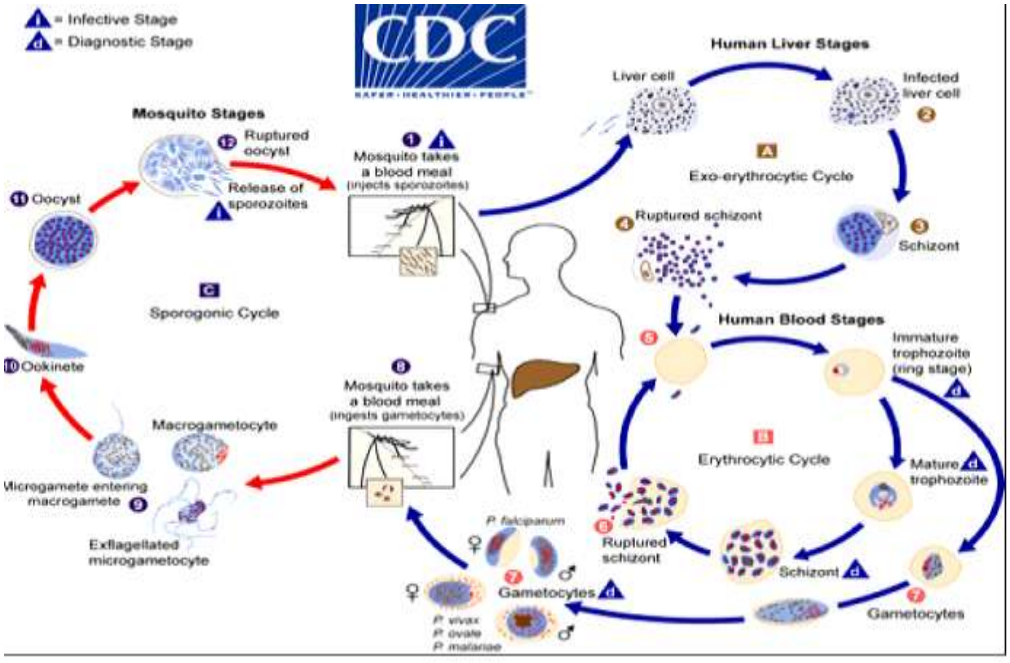


Figure 1: Life cycle of ***Plasmodiumfalciparum*** (Source: Centers for Disease Control and Prevention)

The mechanism of malaria parasite invasion of the human erythrocyte:

The apicomplexan cell entry involves active invasion using an actin-myosin motor involving myosin treadmilling on polymerized actin connected to apicomplexan surface ligands causing an inward movement of the pathogen without a response or involvement of the host cell , endoplasmic reticulum (Koch M and Baum J, 2016). Erythrocyte invasion occurs through a complex multistage process involving numerous parasite host protein interactions, with varying levels of redundancy (Paul et al., 2015), secretion of invasion associated parasite organelles and formation of the unique parasitophorous vacuole within which the parasite resides and develops (Lingelbach and Joiner, 1998).

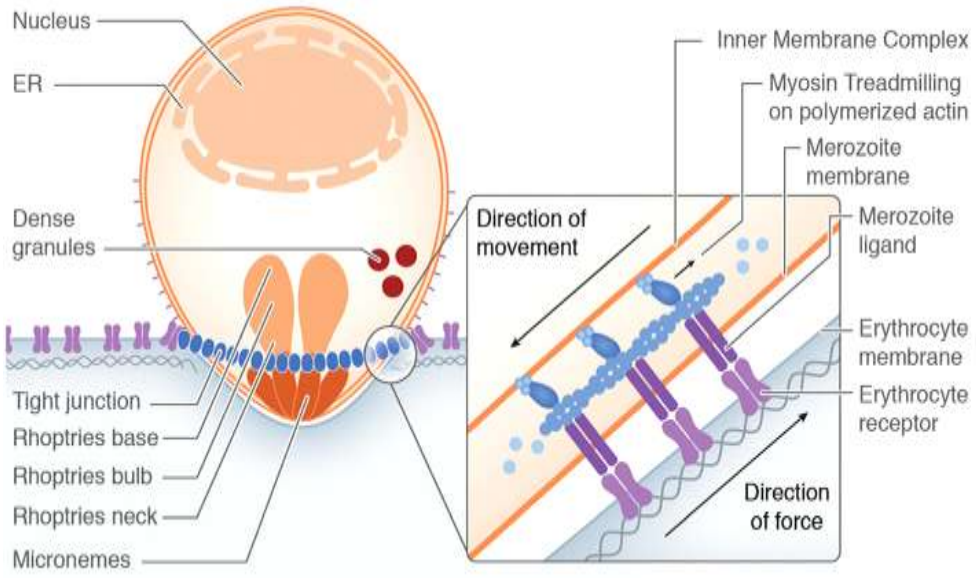


Figure 2: The model for apicomplexan cell entry (Koch M and Baum J, 2016).

The rapid process of invasion is traditionally broken down into four distinct stages (Koch M and Baum J, 2016) , that are,

a) Initial merozoite attachment.
b) Re-orientation of the merozoite to its apical end.
c) Formation of a tight or moving junction.
d) Complete invasion and sealing of the parasitophorous vacuole.

Initial attachment of the merozoite to the erythrocyte can occur in any orientation.

The merozoite then re-orientates itself to position its apical pole in close contactwith the host cell membrane. Numerous parasite host protein interactions occur atthis point particularly, the interactions of EBA175 and Rh4 with their respective host receptors GPA and CR1, which appear to trigger erythrocyte responses results in membrane deformations. Translocation of the RON complex (blue) across the erythrocyte membrane causes establishment of the tight junction, through which the merozoite enters the host cell into a parasitophorous vacuole utilizing force from its actin– myosin motor.

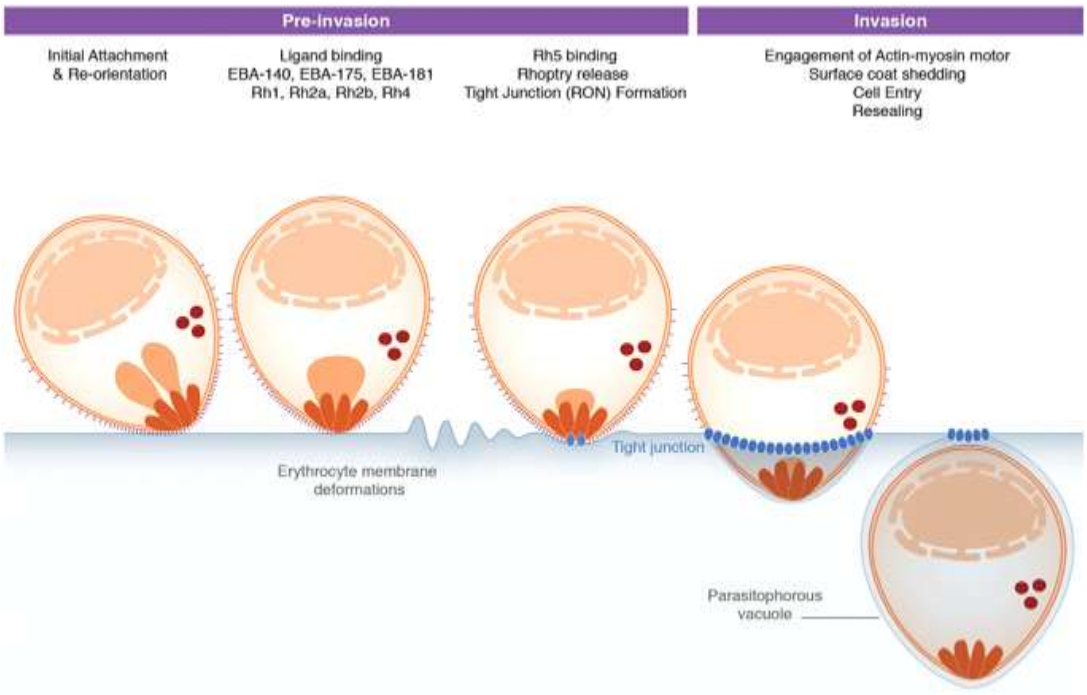


Figure 3: Overview of erythrocyte invasion by *Plasmodium falciparum* merozoites. (Koch M and Baum J, 2016)

The subcellular structure of a *P. falciparum* merozoite consisting the microneme and rhoptry organelles at the apical end is shown in Fig 4 (A). Invasion of the merozoite also involves an initial attachment, which may involve MSPs or directly with EBAs and *Pf*Rh proteins. Apical reorientation is likely to involve membrane wrapping so that the apical end is adjacent to the erythrocyte, allowing tight attachment.

Evidence suggests that a pore is formed between the merozoite and erythrocyte that is mediated either directly or indirectly by the *Pf*Rh5/*Pf*Ripr/CyRPA complex. This is associated with movement of the RON complex on the host membrane. A tight junction is formed involving high-affinity ligand-receptor interactions between AMA1 on the merozoite surface and RON2 inserted in the erythrocyte membrane. This tight junction then moves from the apical to posterior pole powered by the parasite's actinomyosin motor. The surface coat is shed at the moving junction by a serine protease, or ''sheddase.'' Upon reaching the posterior pole, the adhesive proteins at the junction are also proteolytically removed by a resident protease, most likely a rhomboid, in a process that facilitates resealing of the membrane. By this process the parasite does not actually penetrate the membrane, but invades in a manner that creates a parasitophorous vacuole (Koch et al., 2017).

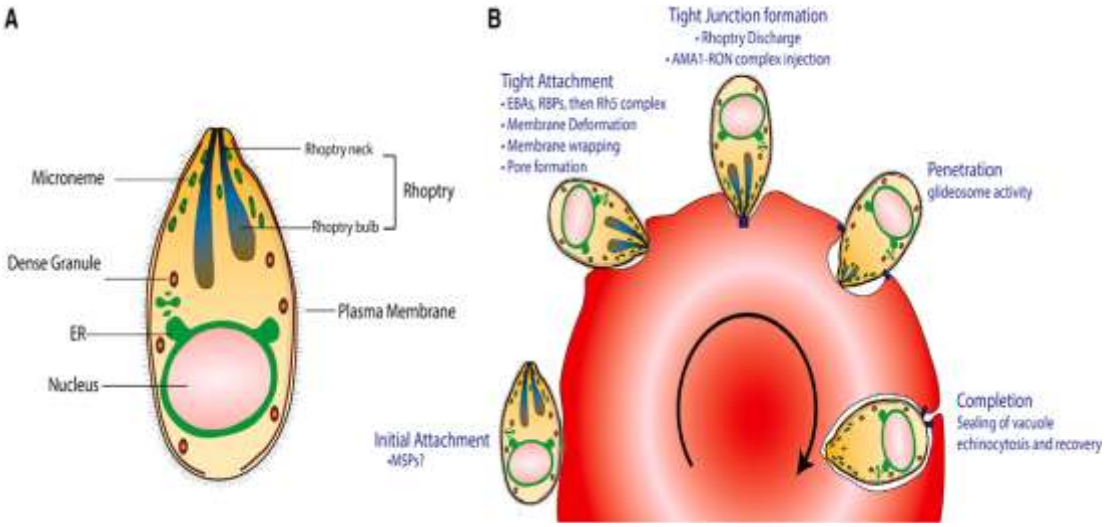


Figure 4: (A) and (B) Structure of the Merozoite and Steps in its Interaction with the Host Erythrocyte (Koch et al., 2017).

Signs and symptoms due to the infection**:**The hallmark symptom of malaria is paroxysm which lasts for one to two hours and occurs in three stages of chills and shivering, followed by high fever and then causing excessive sweating, after which the temperature drops to normal. The fever is induced when the merozoites are released from erythrocytes. The high fever causes vasodilation and hypotension. Erythrocyte destruction leads to anaemia, capillary haemorrhage and thrombosis. Hepatomegaly and splenomegaly occurs due to increased number of macrophages. Other symptoms include headaches, night sweats, dizziness, abdominal pain, nausea, vomiting, mild diarrhoea, tachycardia, muscle pain, fatigue and so on.

Malaria is of two types - benign and malignant. The malignant form of malaria causedby ***P. falciparum***is very severe and sometimes fatal. The fever occurs daily at first, followedby every third day. Whereas the benign forms of malaria are less severe and are causedby *P. ovale, P. vivax and P. malariae.* Here the fever reappears every third day, except in *P.malariae*, where it occurs on every fourth day.

Need for the development of antimalarial dug discovery:

Although reports of antimalarial drug resistance emerged as early as 1910 from South America, the first event that really had a major impact on malaria control and drug development was the emergence of Chloroquine resistance in the second half of 20th century, for which, there has been a marked onset of a race between the development of new generations of antimalarial drugs.

The resistance of the malaria parasites to certain drugs has highlighted the need for the search of novel antimalarial molecules. For the development of an effective antimalarial drug the focus mainly lies in the promising targets. Basic metabolism and biochemical process in the malaria parasite; *Plasmodium falciparum* play an indispensable role in the identification of these targets.

An overview of the enzyme Thioredoxin Reductase : Diversity and Evolutionary significance

Thioredoxin reductases (TrxRs) are the enzymes known for reducing thioredoxin (Trx). They catalyze the transfer of reducing equivalents from reduced substrates to oxidized Trxs. Two classes of thioredoxin reductase have been identified: one class in bacteria and some eukaryotes and one in animals Both classes are flavoproteins functioning as homodimers. Each monomer contains a FAD prosthetic group, a NADPH binding domain, and an active site containing a redox- active disulphide bond. In bacteria, fungi, plants and some protozoan parasites such as *Trichomonas vaginalis*, the protein is 35 kDa; whereas in mammals, insects and Plasmodium falciparum, TrxR is between 55 and 60 kDa (Williams CH et al., 2000).

Members of Trx family differentiated during evolution, are divided into two types based on the attached cofactor:

1) ferredoxin:thioredoxin reductases (FTRs), which are iron-sulfur enzymes; and (ii) flavin-thioredoxin reductases, that are flavoenzymes with a non-covalently bound flavin adenine dinucleotide (FAD).
2) FTRs are heterodimeric enzymes composed of a catalytic subunit (FTRc), containing a 4Fe- 4S center and a redox- active disulfide bond and a variable structural subunit. FTRc interacts with Fdx and Trx to form a ternary complex to deliver electrons from Fdx to Trx (Dai S et al., 2007). FTRs are present in most cyanobacteria and plastids (Droux M et al., 1987).
3) Flavin thioredoxins are a heterogenous group of enzymes with non- covalently bound FAD and are classified based on the reduced donor substrate, that is, NADPH. NTRs or NADPH- dependent Trxs are structurally and mechanistically divided into two main groups that have followed different evolutionary paths (Williams CH et al., 2000). Low molecular weight (LMW)- NTRs and High molecular weight (HMW)- NTRs respectively, containing about 35 KDa and 55 KDa.

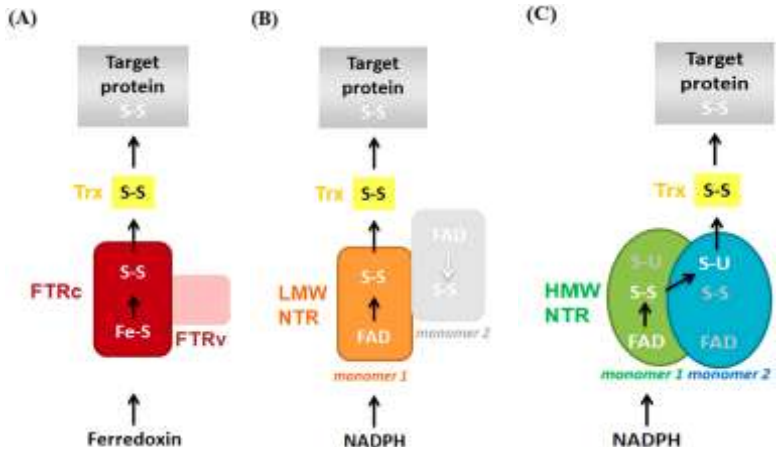


Figure 5: Physiologic reduction of thioredoxins. (A) Heterodimeric ferredoxin:thioredoxin reductase (FTR) (Dai S et al., 2000), (B) Each monomer of the low-molecular-weight NADPH dependent thioredoxin reductase (LMW-NTR) homodimers (Williams CH et al., 1995), (C) High-molecularweight NADPH-dependent thioredoxin reductases (HMW-NTRs) homodimers (Zhong L et al., 2000).

LMW- NTRs are homodimeric enzymes with FAD and a redox active CxxC motif per monomer (CH Williams Jr et al., 1995). They are present in plants, prokaryotes and yeast. This enzymes have evolved diverse structural and catalytic properties and react with different substrates namely NADPH, Fdx, the deazaflavin F420 and unknown (X) (Florencio F et al., 2006). HMW- NTRs are also homodimeric enzymes with three active sites, FAD , a disulfide in the form of CxxxxC, and a redox- active group with a Sec amino acid or a Cys residue that is capable of selenosulfide bond formation (Zhong L et al., 2000). The enzyme is present in most eukaryotes, except yeast and plants.

Also FFTR (ferredoxin- dependent flavin thioredoxin reductase) was discovered more than 35 years ago in the nitrogen- fixing anaerobe *Clostridium pasteurianum* (Hammel K E et al., 1983). This enzyme is similar in molecular properties with NTRs but like FTRs these are reduced by ferredoxin . Another thioredoxin enzyme known as DTR (deeply- rooted TR) was recognized in ancient cyanobacterium *Gloeobacter violaceus*. It consists of modular structures similar to LMW- NTRs and FFTR. The enzyme is found in several types of bacteria, including Aquificace, Chloroflexi, Bacillus, Firmicutes, Chlorobi, Nitrospirae, and Cyanobacteria as well as a few marine algae (Buey R M et al., 2018).

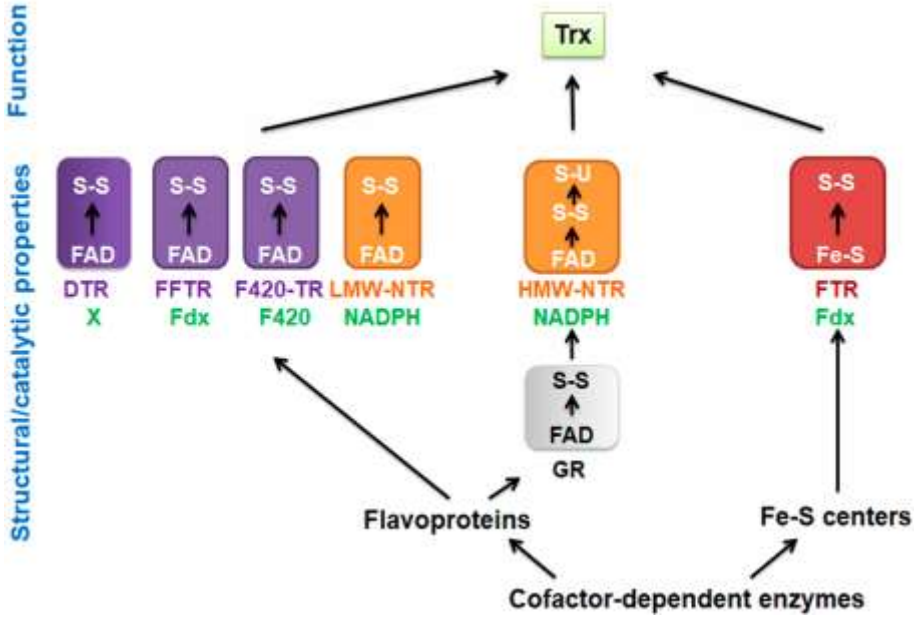


Figure 6: Diversity and evolution of thioredoxin reductases (Florencio F et al., 2006).

Mammalian TrxR can be found as three isoenzymes: TrxR1, TrxR2, and TGR. hTrxR1 is the cytosolic form of the enzyme while hTrxR2 is the mitochondrial form. Both HTrxR1 and HTrxR2 have the CVNVGC

active site and the FAD- binding domain and NADPH- binding domain common to Plasmodium TrxR forms. However, hTrxR2 has a 33- amino acid extension at the N- terminus which is thought to be a mitochondrial translocation signal. TGR also known as hTrxR3, exists mainly in developing sperm. It has a N- terminal glutredoxin domain, hence the name " thioredoxin glutathione reductase" or TGR (Muller S et al., 2003). Like hTrxR, *Pf*TrxR has also an isoform found in the cytosol (*Pf*TrxR1) and one found in the mitochondria (*Pf*TrxR2) (Boumis G et al., 2012).The amino acid sequences of TrxR from different *Plasmodium spp* obtained from Gene Bank reveals about a 40% sequence identity of *Pf*TrxR to the longer hTrxR2 and a 42% identity to the shorter hTrxR1. It has a 77%–80% identity to the five other listed *Plasmodium*species(Table 1). The TrxR in *P. berghei* has been localized to the cytosol (Su D et al.,2005).. This could explain its slightly greater identity to hTrxR1 than hTrxR2. The CVNVGC active site is conserved for all six species of *Plasmodium* and human isoenzymes. Conversely, the GCGGGKC C- terminal is conserved in all six *Plasmodium* species but is not present in the human isoenzymes. Sequences of TrxR for *P. falciparum* (*P. falc.*), *P. vivax* (*P. viv*), *P. yoelli yoelli* (*P. yoel.*), *P. berghei* ANKA (*P. berg*), *H. sapiens* 1 (*H. sap1*), *H. sapiens* 2 (*H. sap*2), *P. cynomolgi* strain B (*P. cyn*), and *P. knowlesi* strain H (*P. know*) obtained from the National Center for Biotechnology Information (NCBI) protein databank are shown in Table 1.

Table 1: Amino acid sequence identities between *Plasmodium* sp. TrxRs, the human cytosolic and mitochondrial isoforms. (As obtained from the National Center for Biotechnology Information (NCBI) protein databank)**.**

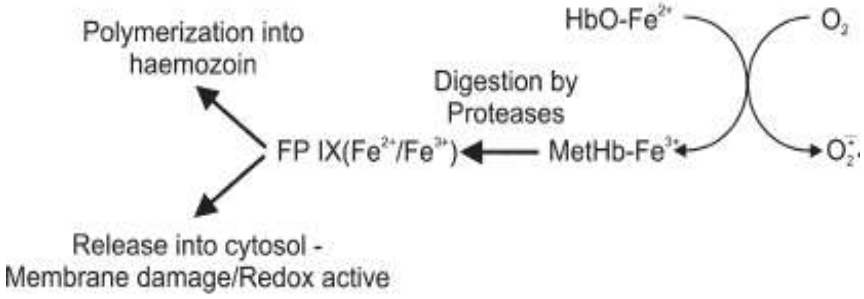
	H. sapiens **(hTrxR1)**	***H. sapiens*** **(hTrxR2)**	***P. falciparum***	***P. vivax***	***P. yoelii yoelii***	***P. berghei*** **ANKA**	***P. knowlesi*** **Strain H**	***P. cynomolgi*** **Strain B**
Accession Number	AAB35418	AAD19597	CAA60574	EDL45043	EAA21839	XP_679935	XP_002258509	XP_004221759
Number of amino acids	497	524	541	546	638	542	623	628
Identity to *P. falciparum* (%)	42	40	100	77	79	79	79	80
Identity to *H. sapiens* (hTrxR2) (%)	54	100	40	41	41	40	41	41
Identity to *H. sapiens* (hTrxR1) (%)	100	54	42	41	42	42	41	41

Why Thioredoxin reductase is a potential target for novel antimalarial drug development ?

Thioredoxin reductase of *Plasmodium falciparum* is the only *Plasmodium* TrxR that has been crystallized since *P. falciparum* is the species responsible for most of the malaria cases. The malaria parasite *Plasmodium falciparum* is highly adapted to cope with the oxidative stress to which it is exposed during the erythrocytic stages of its life cycle. This includes the defence against oxidative insults arising from the parasite's metabolism of haemoglobin resulting in the formation of reactive oxygen species. The parasite contains a functional Thioredoxin reductase system which is essential for the survival of their intraerythrocytic stages. The intracellular levels of ROS are kept at a low level by thioredoxin which are actually redox messengers interacting with redox active proteins like thioredoxin reductase. The biological functions of thioredoxin are essential for parasite survival and the knockout of thioredoxin reductase will have a lethal effect on the parasite.

The Antioxidant system in *Plasmodium falciparum* :

Infection with *P. falciparum* leads to increased oxidative stress in red blood cells, because when the haemoglobin is taken up by the parasites into their acid food vacuole the spontaneous oxidation of Fe^{2+} to Fe^{3+} leads to the formation of superoxide ions which inevitably leads to the generation of hydrogen peroxide and subsequently hydroxyl radicals, both highly reactive and toxic oxygen intermediates (Liochev and Fridovich, 1999).



(**Source: Muller S, 2004**)

$$O_2^- \xrightarrow{SOD} H_2O_2 + O_2$$
$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

To combat the oxidative stress so produced the parasite must have an efficient antioxidant removal system to prevent damage caused by reactive oxygen species. The effect is primarily achieved by a functional low molecular weight thiol-thioredoxin system utilizing *Pf*TrxR (McCarty S et al., 2015). The activity of the enzyme is essential for an adequate intracellular redox environment during intraerythrocytic development and proliferation, and consequently is necessary for the survival of the parasites (Gilberger T.W. et al., 2000).
Plasmodium falciparum thioredoxin reductase (PfTrxR) is between 55 and 60 kDa (Williams et al., 2000; Coombs et al., 2004) containing three redox centre: FAD as a prosthetic group and two cysteine-based redox centres which catalyze the following reaction:

$$\boldsymbol{NADPH + H^+ + Trx - S_2 \rightarrow NADP^+ + Trx - (SH)_2.}$$

The electrons are shuttled from NADPH via FAD to the N-terminal active site (Cys88 and Cys93). Subsequently, the electrons are transferred to the C-terminal redox motif (Cys535 and Cys 540) that finally reduce the substrate thioredoxin (*Pf*Trx) (Gilberger TW et al., 1997).

The dimer interface of *Pf*TrxR is marked by a diagonal line (Fig 7). Each monomer binds NADPH + H^+, and the electrons are transferred to FAD and subsequently to the N-terminal redox center (Cys88 and Cys93). Cys88 attacks Cys540 in the interchange reaction between the N-terminal and C-terminal cysteine-based redox centers.

Cys540 performs an nucleophilic attack on the disulfide of the substrate thioredoxin (*Pf*Trx1), resulting in a mixed disulfide between Cys540 ^{PfTrxR} and Cys30 ^{PfTrx1}. The mixed disulfide is finally resolved by Cys535 ^{PfTrxR}, thereby releasing the reduced substrate.

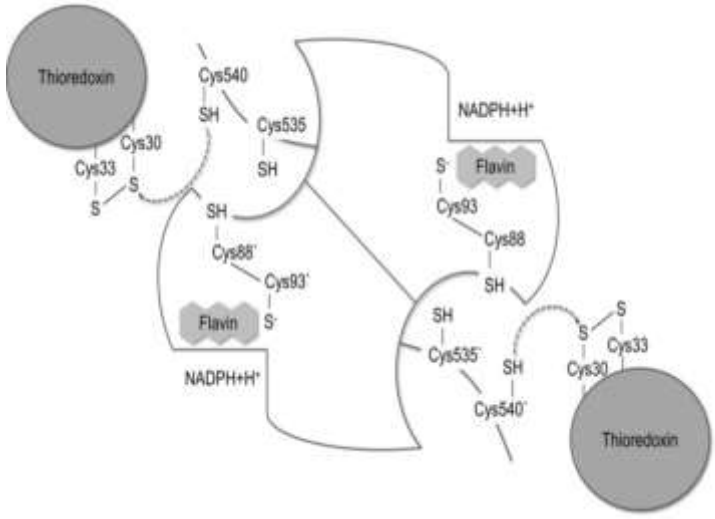


Figure 7: Scheme of the reduction of the substrate thioredoxin (*Pf*Trx1) by thioredoxin reductase (*Pf*TrxR) (Fritz-Wolf K et al., 2013).

The *P. falciparum* TrxR gene has been genetically validated as a potential drug target as its disruption would be lethal for the parasites. (Krnajski et al., 2002).

Structure of Plasmodium falciparum thioredoxin reductase (*Pf*TrxR):

The structure of *Pf*TrxR complexed with its substrate, thioredoxin (Fig 8) shows distinct characteristics. *Pf*TrxR is a homodimeric protein where each subunit divides into three major domains. The FAD-binding domain roughly includes residues 38 to 197 and 321 to 390. The residues between the two sequence components of the FAD-binding domain (i.e., 198-320) is represented as the NADPH-binding domain, and the C-terminal segment of the subunit from about S391 to the C-terminus (G541) is referred to as the interface domain. The homodimeric structure of *Pf*TrxR contains two fully functional pairs of redox centers. The N-terminal redox center is referred to as such because the bulk of residues for FAD and NADPH binding as well as electron transfer associated with this center are provided by the N-terminal domains (38-320) and the two residues provided by the C-terminal interface domain of the second subunit (H509 and E514) contribute to the function of the center. The location of the

N-terminal dual-cysteine redox center is adjacent to the isoalloxazine ring system of the FAD prosthetic group. The C-terminal redox center is accounted for almost entirely by the C-terminal nine amino acids (G533- G541), notably cysteines 535 and 540. An insertion loop also interacts with both the substrate and the enzyme. The flexible conformation of the C-terminal arm and the extended insertion loop set *Pf*TrxR contribute to differences in substrate binding and reduction (Boumis G et al., 2012).

Formation of the enzyme-substrate (*Pf*TrxR-Trx) complex results in rearrangement of the C-terminal active site and the insertion loop, which is a necessary conformation change for the enzyme's catalytic cycle (Fritz- Wolf k et al., 2013)

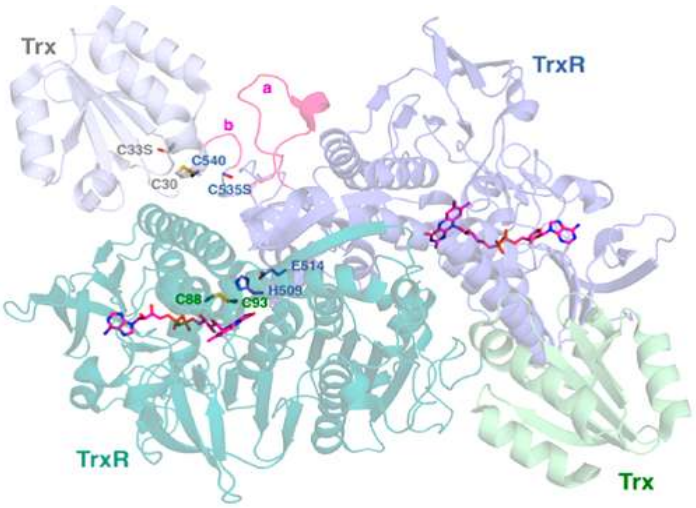


Figure 8: The dimeric structure of *P. falciparum* thioredoxin reductase (*Pf*TrxR). Thetwo subunits of TrxR are shown with their bound FAD cofactors in magenta. The redoxactive disulfide (C88–C93) of the N-terminal redox center is shown for the *Pf*TrxR monomeron the lower left. H509 and E514 that modulate the reactivity of this N-terminal redox centerare supplied by the *Pf*TrxR monomer on the upper right. Each monomer is bound to substrateTrx via intermolecular disulfide (*Pf*TrxR C540 to Trx C30). The positions of C535 (*Pf*TrxR) and C33 (Trx) are indicated by serine residues. These substitutions were made in order to trap the intermolecular disulfide between *Pf*TrxR and Trx (Fritz- Wolf K et al.,2013) .The 535 and 540 residues shown are supplied by the *Pf*TrxR monomer on the upper right. The *Plasmodium*-unique insertions H438–S452 and G536–K539 are highlighted by *a* and *b* respectively (Fritz- Wolf K et al., 2013).

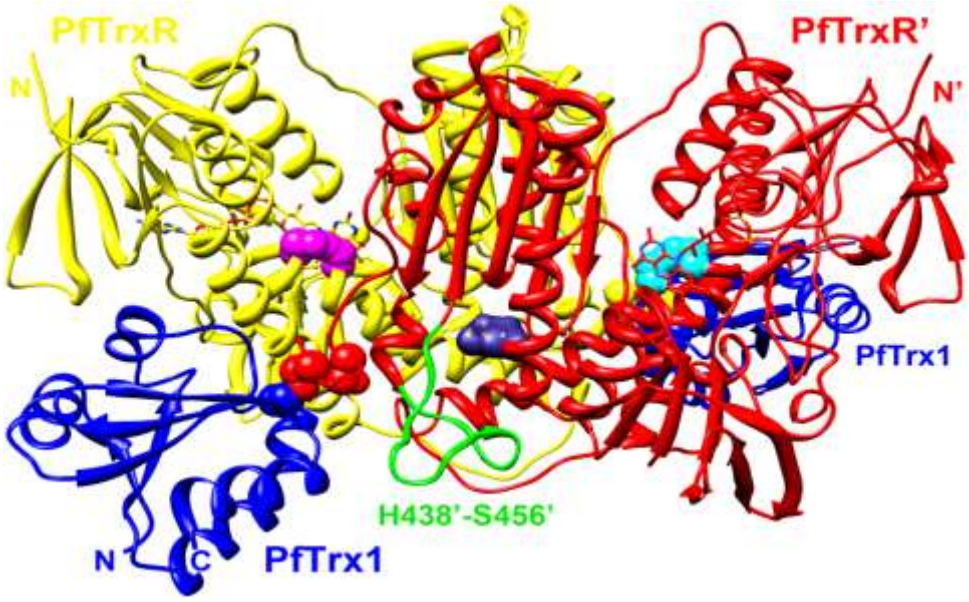


Figure. 9: Overview of the *Pf*TrxR dimer (red/yellow) bound with the substrate *Pf*Trx1 (blue). The redox centers of the yellow subunit are shown with magenta (N-terminal) and yellow (C-terminal) balls. In the red subunit, the redox centers are colored cyan (N-terminal) and red (C-terminal), and in *Pf*Trx1, they are in blue. The intersubunit cavity is indicated in slate blue. The FAD molecules are represented as sticks and the loop region His438–Ser456 of the red subunit is shown in green **(Pettersan et al).**

The key Aspects of *Pf*TrxR Active Sites for Enzyme Inhibition:

N-Terminal Redeox Center:

Four amino acids in the N-terminal domain are essential to TrxR activity: Cys 88, Cys 93, His 509, and Glu 514, Cys 88 and Cys 93. These are buried in the protein and are endpoints of a strictly consvered CVNVGC sequence that forms a single five-amino-acid helical turn (Snider G et al., 2014 and Gilberger T et al., 1997) . When the enzyme is oxidized both Cys 88 and Cys 93 are joined to one another through a disulfide bond which resides above N1 of the flavin isoalloxazine ring system (Fritz- Wolf K et al.,2013).

In case of reducing DTNB if Cys 88 or Cys 93 is replaced with either Ser or Ala, it results in complete loss of TrxR activity. His 509 and Glu 514 are essential to the active site and are contributed by a strongly conserved region of the interface domain of the second subunit. The ε2 nitrogen of the histidine imidazole is in closest proximity to the sulfur of Cys 88. Simultaneously, the δ1 nitrogen of the His 509 imidazole is in H-bonded contact with the Glu 514 carboxylate (Fritz- Wolf K et al., 2013).

Thus, it is proposed that these two residues form a catalytic dyad where the general acid or base properties of His 509 are modulated by Glu 514 (Gilberger T et al., 1997). This is supported by substitution of His 509 with either Gln or Ala which decreases the *Pf*TrxR k_{cat} for NADPH-dependent DTNB reduction by two orders of magnitude with relatively small impact on the apparent K_M values for either substrate. Also, substitution of Glu 514 with Ala produces a decrease in activity (Gilberger T et al., 1997).

C-Terminal Redox Center:

The C-terminal redox center is found on the highly conformationally flexible C-terminus of the interface domain. *Pf*TrxR employs a two cysteine redox pair (Cys 535 and Cys 540) where the Cys residues are separated by an intervening four-amino-acid linker (GCGGGKCG-COO^-) (Boumis G et al., 2012). Several amino acids and a disulfide ring contribute to the active site of the C-terminal redox center. Cys 535 is hydrogen bonded to neighbouring amino acids on the flexible arm of the terminal domain having several important functions including electron transfer, reduction of thioredoxin and serving as the resolving cysteine of the intermolecular disulfide. Cys 540 is also involved in electron transfer and reduction of the substrate, it is been the most physiologically relevant nucleophilic cysteine of the C-terminus due to its flexibility, since it is located on the flexible arm of the C terminus, it is determined to be the ideal nucleophile to approach the substrate (Fritz- Wolf et al., 2013).

The C-terminal amino acid (Gly 541) affects the enzymatic activity by forming a salt bridge with Lys58 with its backbone carboxylate as well as a hydrogen bond between its amide nitrogen and the carbonyl oxygen of Gly 534 (Snider G et al., 2014).

The hydrogen bond is more important in stabilizing the β-turn-β motif and serves as an anchor for the reduction of the C-terminal domain disulfide. The C-terminal redox center has a twenty-member disulfide ring

when C-terminal Cys residues 535 and 540 are oxidized, similar to the N-terminal extension, which has an impact on activity. Deletion of Lys 539 from between the C-terminus cysteines in the ring, leaving it with only seventeen atoms, has a significant reduction on the enzyme activity. Therefore, C-terminal domain chain length is important to the ring's functionality (Snider G et al., 2014).

Enzyme Catalytic Cycle:

TrxR-catalyzed reduction of Trx by NADPH is proposed to require a priming stage where NADPH binding is followed by formation a $FADH^-$ to $NADP^+$ charge transfer complex. Along with $NADP^+$ release, $FADH^-$ is reoxidized at the expense of Cys 93, forming Cys- S^- to FAD charge transfer complex and a thiol for subsequent disulfide exchange at Cys 88. This form of TrxR is proposed to be the start and end of the enzyme's catalytic cycle. Through intersubunit dithiol-disulfide exchange with the disulfide C-terminal redox center, the N-terminal redox center is returned to its fully oxidized state (*i.e.*, FAD, Cys 88–Cys 93 disulfide) and the C-terminal redox center is reduced from its disulfide to dithiol. In a sequence essentially identical to the priming steps, NADPH binding produces the $NADP^+$ to

$FADH^-$ charge transfer complex which again transitions to a Cys 93 thiolate to FAD charge transfer complex and a Cys 88 thiol. With both the N- and C-terminal redox centers fully reduced, Trx reacts with the C-terminal redox center through dithiol: disulfide exchange to convert the Trx disulfide substrate to the corresponding reduced dithiol Trx product.

In so doing, the C-terminal redox center returns to its oxidized disulfide state, and the next reaction cycle can begin (McMillan P et al., 2006).

Interactive Cavity and Interface:

Within *Pf*TrxR there are a narrow active site cavity and a monomer-monomer interface. In the cavity, Tyr 101 and His 104 alter the stereo-chemical properties of the cavity in *Pf*TrxR. The narrowness is suggested to be due to a H-bond with Asp 112 on the α3 helix from the other monomer. Due to the narrowness of the cavity, it is difficult for larger molecules to access the *Pf*TrxR active site. Indeed, it has been suggested that the cavity can host smaller, more amphipathic molecules. The *Pf*TrxR interface is a bent α3 helix because of the unique presence of Met 105 and Phe 109 contributing to dimer stability and to substrate binding of thioredoxin due to the bulky Met 105 and Phe 109 inducing a bend that goes on to stimulate a conformational change downstream on the same interface. *Pf*TrxR assumes an anti-parallel conformation that allows H-bonding particularly between Asp 121 and Asn 122 (Boumis G et al., 2012 ; Fritz- Wolf K et al., 2013).

Insertion loop:

*Pf*TrxR has an insertion loop of amino acids, His 438- Ser 456, in a cleft within the protein consisting of nineteen residues in *Pf*TrxR. Different parts of the loop interact with different substrates or intermolecular residues. Some interact only with residues of *Pf*TrxR, others with the C-terminus in particular, and three interact with reductase and substrate interface residues. Deletion of residues 438–452 of the loop produces a no change in the K_M for the pseudosubstrate DTNB but a seven fold increase in K_M for the true substrate, Trx. Interestingly, the *k*cat value with respect to both substrates were only moderately diminished. These data suggest that the insertion loop is important for interaction and complex formation between *Pf*TrxR and its *Pf*Trx substrate. His 438 may be particularly important in reduction of the disulfide bond. *Pf*TrxR has a higher K_M and lower k_{cat} for both *Pf*Trx and DTNB (Fritz- Wolf K et al., 2013).

The Inhibitory Activity toward *Pf*TrxR: Screening for mechanism of action

Medicines for Malaria Venture (MMV) has pre-screened and identified a set of 400 antimalarial compounds called the Malaria Box. From those, about five compounds have been evaluated according to their mechanisms of action through inhibition of *Pf*TrxR Five compounds with significant *Pf*TrxR inhibitory activity, with IC_{50} values ranging from 0.9–7.5 μM against the target enzyme, were found out of the Malaria Box (Munigunti R et al., 2013)

In order to catalyze the development of new antimalarials, MMV and the pharmaceutical company SCYNEXIS assembled the Malaria Box, an accessible collection of 400 non-toxic natural and synthetic chemotypes identified by phenotypic screening for asexual intraerythrocytic stages of *Plasmodium falciparum* (Bowman JD et al., 2013). Half of these compounds were selected based on their drug-like properties and the others as molecular probes. The mechanisms of action and the activity of these compounds in other stages of the parasite's life cycle remain to be determined which make the set of compounds worth exploring for drug discovery. Promising molecules with new mechanisms of action will catalyze drug development and are essential in combating growing antimalarial resistance.

Two hundred and fifty compounds were screened against *Pf*TrxR at 10 μM due to their inclusion in the Malaria Box, indicating their established antimalarial activity and chemical diversity. By using a pre-established functional assay (Munigunti R et al., 2013) five compounds (Fig 10) showed *Pf*TrxR inhibition greater than 50% at 10 μM and were selected for IC_{50} value determination. These compounds are shown below:

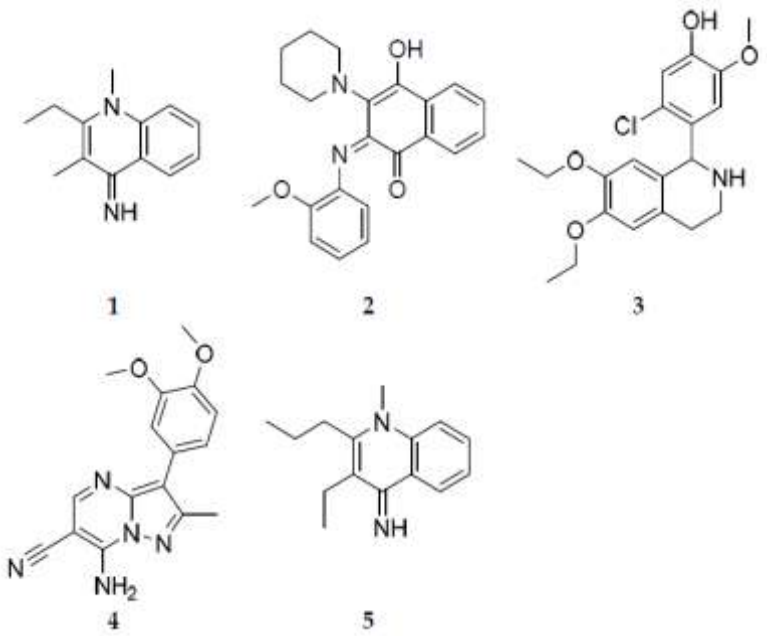


Figure 10: Structures of *Pf*TrxR inhibitors (1-5) (Munigunti R et al., 2013)

Table 2: Identification of the five active *Pf*TrxR inhibitors (Munigunti R et al., 2013).

Compound Number	MMV	IUPAC Name
1	MMV006278	2-ethyl-1,3-dimethylquinolin-4-imine;hydroiodide
2	MMV085203	2-(2-methoxyanilino)-3-piperidin-1-ylnaphthalene-1,4-dione
3	MMV008956	5-chloro-4-(6,7-diethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-2-methoxyphenol
4	MMV396797	7-amino-3-(3,4-dimethoxyphenyl)-2-methylpyrazolo[1,5-a]pyrimidine-6-carbonitrile
5	MMV008416	3-ethyl-1-methyl-2-propylquinolin-4-imine;hydron;iodide

Compound2 was the best inhibitor of the target and most active against *Pf*3D7. It showed an IC_{50} of 0.9 μM against the target enzyme compared to the 3.5–7.5 μM of the other compounds so tested (Munigunti R et al., 2013).

The IC_{50} value of **compound1**, however, is nearly double that of **compound5** which indicates that the extra carbons increase cyto-toxicity. (Munigunti R et al., 2013).

Bis-(2,4-dinitrophenyl) sulfide (2,4-DNPS), a standard inhibitor for the *Pf*TrxR enzyme, was also evaluated against *Pf*TrxR as a positive control Another specific inhibitor of *Pf*TrxR namely 6,7-dinitroquinoxaline demonstrating a mechanism of uncompetitive inhibition of *Pf*TrxR as a nitrophenyl derivative was also identified (Munigunti R et al., 2012).

6,7-Dinitroquinoxaline is also proved to be active in the lower micromolar range on the chloroquine-resistant *P. falciparum* strain in vitro (Andricopulo A D et al., 2006). Other inhibitors of *Pf*TrxR namely, 1,4-napthoquinone, 4-nitrobenzothiadiazole and menadione with antimalarial activity have also been described by Munigunti et al.

Animal Models Available to Test PfTrxR Inhibitors:

Murine models, or mice models, are the most commonly used first step in *in-vivo* malaria drug testing, despite the apparent limitations of truly reflecting a malaria infection within a human (Kaira B et al., 2006). Mice are nonprimates that are normally unable to be infected by the *Plasmodium* species that affect humans. In one mouse model by Badell, *et al.* the (**bg/bg xid/xid nu/nu**) BXN mice were immunocompromised in order to sustain *P. falciparum*-parasitized human erythrocytes *in-vivo*. The group experimented using different methods to best suppress the immune response of the mouse to sustain AB human red blood cells and parasitemia using sub-lethal irradiation with cyclophosphamide or injecting di-chloromethylene biphosphonate-(CI, MBP)-encapsulated liposomes which target tissue macrophages and anti-PMN monoclonal antibodies in the mouse. A combination of the two had resulted in parasitemia of 3%, but values fluctuated greatly as the immune response still reacted despite measures to suppress or the mouse died (Badell E et al., 1995). This presents that *P. falciparum* can be modeled promisingly in mice and, therefore, vaccines and antimalarials can be tested. Murine models could potentially also be used to model *P. vivax* (Kaira B et al., 2006). Identification of mefloquine ,halofantrine (Peters W et al., 1977)**,** and artemisinin derivatives as antimalarials (Vennerstrom, J.L. et al., 2000)was done through use of rodent models, indicating that there is correlation and ability to predict the response to treatments by infected humans

Use of a primate model after a murine model confirms the results of drug and/or vaccine efficacy in rodents and is a better prediction of any effects on humans. The owl monkey, *Aotus trivirgatus*, and *Saimiri* primate models are capable of sustaining *P. falciparum* and *P. vivax* infections. These species are well characterized and provide a more clear relationship between human infection and antimalarial response than in murine models, making the use of primate models the stage before clinical trials (Fidock D et al., 2004 ; Moll K et al., 2008) for antirelapse radical curative activity against hyponozoites (Deye GA et al.,2012).

Choice of animal model depends upon cost per test, rate of testing needed, reproducibility of efficacy of the antimalarial, the known sensitivity of the drug, and other factors relevant to the research.

Future Prospects For Studies:

The overview on *Pf*TrxR presented above clearly underline the necessity of Trx related functions for survival of the *P. falciparum* within the host erythrocytes. Further testing on whether or not viable parasites of *P. falciparum* could be created under similar thioredoxin reductase (TrxR) knockout conditions is needed to provide a more definitive answer as to whether or not TrxR is essential for the survival of the human and rodent *Plasmodium* species. Further TrxR knockout testing with other *Plasmodium* strains would also be useful in understanding the essentiality of TrxR across *Plasmodium* species. The crystal structure has been determined only for *P. falciparum*; therefore, crystallization of the TrxRs of other *Plasmodium* species could provide information about structural and active site similarities between *Plasmodium* species and the human form. Also, the crystal structure of *Pf*TrxR may be used as a template to determine structures of TrxR from other malaria species by homology modeling. If there would be any striking similarities and sequence homologies between TrxR form in different malaria species found then it would be important and could also indicate that the rodent malaria model would be suitable testing the inhibitors of *Pf*TrxR with potent antimalarial activity *in vitro* in phenotypic *P. falciparum* screening.

III. Conclusion:

Plasmodium inhabits the human erythrocytes for a fraction of its intricate life- cycle. The pathophysiology of malaria is caused by the developmental stages of *Plasmodium* and these stages are targeted for effectual interventions for antimalarial drugs. An oxidative stress is known to occur in *Plasmodium* infected erythrocytes which render them vulnerable to oxidative challenge. In order to thrive, the parasite requires efficient antioxidants to shield itself against damages like nucleic acid modifications, lipid peroxidation, and oxidation of thiol containing proteins, caused by ROS. Thus an approach to disrupt the parasite's antioxidant system by focusing on redox systems in *Plasmodium* holds promise for inhibiting the development and survival of the parasite in the host cell.

*Pf*TrxR (Thioredoxin reductase), belonging to the family of dimeric flavoenzymes, include lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase reduces thioredoxin (Trx). Inhibition of this enzyme is likely to affect the parasite at several vulnerable points resulting in enhanced oxidative stress, ineffective DNA synthesis and cell division and distributed redox regulatory processes. The significant structural and mechanism of *Pf*TrxR makes it a promising target.

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