MOLECULAR CLONING, EXPRESSION, AND CHARACTERIZATION OF GLUTATHIONE-S-TRANSFERASE AS A NOVEL TARGET IN ANTI-MALARIAL DRUG DESIGN AND DISCOVERY

By

MOHAMMED NOORALDEEN MAHMOD
AL-QATTAN

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To all Muslim brothers:

"Allah will deprive usury of all blessing, but will give increase for deeds of charity" (Quran 2: 276). In a hadith narrated by Abu 'Amir or Abu Malik Al-Ash'ari: that he heard the Prophet saying, "From among my followers there will be some people who will consider illegal sexual intercourse, the wearing of silk, the drinking of alcoholic drinks and the use of musical instruments, as lawful. And there will be some people who will stay near the side of a mountain and in the evening their shepherd will come to them with their sheep and ask them for something, but they will say to him, 'Return to us tomorrow.' Allah will destroy them during the night and will let the mountain fall on them, and He will transform the rest of them into monkeys and pigs and they will remain so till the Day of Resurrection." (No. 5226, Sahih Al-bukhari).

Allah’s wisdom ruled that sins do not provide richness and development even if sin-fullers pretend to have it. Furthermore, pretending to have what you have not is a sin. It is related from Asma' from the Prophet (may Allah bless him and grant him peace) that a woman said, "Messenger of Allah, I have a co-wife. Would it be a sin if I were to pretend to have received something from my husband which he has not given me?" The Prophet said, "Anyone who pretends to have received something he has not been given is like someone who wears two spurious garments [i.e. someone who dresses up to give a false impression]." (No.4921, Sahih Al-bukhari)

Attending the path of disbelievers seeking for development and reputation is an attribute of Monafiqin and soon they will blame themselves when ALLAH give victory to believers. {O you who have believed, do not take the Jews and the Christians as allies. They are [in fact] allies of one another. And whoever is an ally to them among
you - then indeed, he is [one] of them. Indeed, Allah guides not the wrongdoing people
(51) So you see those in whose hearts is disease hastening into [association with] them,
saying, "We are afraid a misfortune may strike us." But perhaps Allah will bring
conquest or a decision from Him, and they will become, over what they have been
concealing within themselves, regretful (52)} (Ayah 51-52, Surah Al-Maidah).

The beneficial knowledge is really that which descended from ALLAH regarding His names, actions and attributes. {So turn away from whoever turns his
back on Our message and desires not except the worldly life (29) That is their sum of
knowledge. Indeed, your Lord is most knowing of who strays from His way, and He
is most knowing of who is guided (30)} (Surah Al-Najm ayah 29-30)

Other knowledge even beyond quantum level is merely superficial and will not
prevent punishment if it comes. {Have they not traveled through the land and observed
how was the end of those before them? They were more numerous than themselves
and greater in strength and in impression on the land, but they were not availed by
what they used to earn (82) And when their messengers came to them with clear proofs,
they [merely] rejoiced in what they had of knowledge, but they were enveloped by
what they used to ridicule (83) And when they saw Our punishment, they said," We
believe in Allah alone and disbelieve in that which we used to associate with Him."
(84) But never did their faith benefit them once they saw Our punishment. [It is] the
established way of Allah which has preceded among His servants. And the disbelievers
thereupon lost [all] (85)} (Ayah 82-85, Sura Ghafir).

The prophet Mohammed (PBUH) said “Does not thank Allah the person who
does not thank the people” (No. 211, Al-adab Al-mofrad for Bukhary). Therefore, I
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<tr>
<th>Symbol</th>
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<tr>
<td>( \Delta A_{\text{blank}} )</td>
<td>Change in absorbance for blank</td>
</tr>
<tr>
<td>( \Delta A_{\text{sample}} )</td>
<td>Change in absorbance for sample</td>
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<tr>
<td>( \Delta G_{\text{GB}} )</td>
<td>Total free energy using MMGBSA (GBTOT-TSTOT)</td>
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<tr>
<td>( \Delta G_{\text{PB}} )</td>
<td>Total free energy using MMPBSA (PBTOT-TSTOT)</td>
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<td>([E])</td>
<td>Enzyme concentration</td>
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<tr>
<td>IC(_{50})</td>
<td>The concentration produces 50% inhibition</td>
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<tr>
<td>( K_{\text{cat}} )</td>
<td>Rate constant for enzyme catalysis</td>
</tr>
<tr>
<td>( K_d )</td>
<td>Rate constant for dissociation of enzyme-inhibitor complex</td>
</tr>
<tr>
<td>( K_i )</td>
<td>Rate constant for enzyme inhibition</td>
</tr>
<tr>
<td>( K_{iCDNB} )</td>
<td>Inhibitory constant for enzyme inhibition measured by varying inhibitor and CDNB concentrations</td>
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<tr>
<td>( K_{iGSH} )</td>
<td>Inhibitory constant for enzyme inhibition measured by varying inhibitor and GSH concentrations</td>
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<td>( K_m )</td>
<td>Concentration of substrate that occupied 50% of enzyme binding sites.</td>
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<td>Concentration of CDNB to achieve half of maximum enzyme velocity</td>
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<tr>
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<tr>
<td>( V_{\text{max}} )</td>
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<tr>
<td>( v_o )</td>
<td>Maximum enzyme velocity</td>
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### LIST OF ABBREVIATIONS

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>AM1-BCC</td>
<td>Semi-empirical (AM1) with bond charge correction (BCC)</td>
</tr>
<tr>
<td>A. paniculata</td>
<td>Andrographis paniculata</td>
</tr>
<tr>
<td>B. purpurea</td>
<td>Bauhinia purpurea</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair. Usually used for DNA size</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C</td>
<td>Cytosine</td>
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<td>Croton argyratus</td>
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<td>CB</td>
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<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DFR</td>
<td>Docked-Fragment Replacement</td>
</tr>
<tr>
<td>dH2O</td>
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</tr>
<tr>
<td>DMSO</td>
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</tr>
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<td>dNTP</td>
<td>Deoxynucleotide phosphate</td>
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<tr>
<td>DSPT</td>
<td>Disulphophenyl triazine</td>
</tr>
<tr>
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<td>Eurycoma longifolia</td>
</tr>
<tr>
<td>EA</td>
<td>Ethacrynic acid</td>
</tr>
<tr>
<td>ELE</td>
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<td>Guanine</td>
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<td>Reaction field energy calculated by GB</td>
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<td>Hydrophobic contribution to total free energy (GBSUR + VDW)</td>
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<td>GBSOL</td>
<td>Desolvation energy (GBSUR + GBCAL)</td>
</tr>
<tr>
<td>GBSUR</td>
<td>Hydrophobic contrib. to solv. free energy for GB calculations</td>
</tr>
<tr>
<td>GBTOT</td>
<td>Total enthalpy contribution of free energy (GBSOL + GAS)</td>
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<tr>
<td>GS-DNB</td>
<td>S-(2,4-dinitrobenzyl) glutathione</td>
</tr>
<tr>
<td>GS-EA</td>
<td>Glutathione-ethacrynic acid conjugate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>G-site</td>
<td>Glutathione binding site</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GSX</td>
<td>S-hexyl glutathione</td>
</tr>
<tr>
<td>h</td>
<td>Hill slope</td>
</tr>
<tr>
<td>H</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hGSTP1</td>
<td>Human Glutathione-S-transferase Pi-1</td>
</tr>
<tr>
<td>HOMO</td>
<td>Higher occupied molecular orbital</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>H-site</td>
<td>Hydrophobic binding site</td>
</tr>
<tr>
<td>IFR</td>
<td>Isosteric-Fragments Replacement</td>
</tr>
<tr>
<td>Inter MIE</td>
<td>Inter-Molecular Interaction energy (or GAS)</td>
</tr>
<tr>
<td>Intra MIE</td>
<td>Intra-Molecular Interaction Energy (or INT)</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>U</td>
<td>International unit for enzyme activity represent number of µM of product formed per minute per ml (µM/min/ml)</td>
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PENGKOLONAN MOLEKUL, UNGKAPAN DAN PENCIRIAN GLUTATHIONE-S-TRANSFERASE SEBAGAI SASARAN NOVEL BAGI REKABENTUK UBAT-UBATAN DAN PENEMUAN ANTIMALARIA

ABSTRAK

Glutation-S-transferase (GSTs) adalah sekumpulan enzim detoksifikasi. Plasmodium falciparum mempunyai isoform tunggal GST (PfGST) yang terlibat dalam bagi detoksifikasi heme. Isoform GSTs daripada manusia (hGSTP1) dan tikus (mGSTM1) terlibat dalam tekanan apoptosis laluan kinase dan menjadi pengantara bagi kerintangan sel kanser terhadap kemoterapi. PfGST, hGSTP1 dan mGSTM1 telah berjaya diklon dan diungkapkan secara heterologus dalam E. coli. Eksperimen perencatan, kinetik dan penghabluran enzim telah dijalankan untuk mencari sebatian yang berpotensi untuk merencat PfGST. Substrat P/GST iaitu glutation (GSH) dan 1-kloro-2,4-dinitrobenzena (CDNB), serta perencat glutation yang diketahui iatu S-hexyl (GSX), cibacron biru (CB), asid etacrinic asid (EA), Hemin, protoporfirin IX (protoIX) dan 4- (2-hidroxietil) -1 asid-piperazineetanesulfonik (HEPES) telah dikaji. Keputusan menunjukkan afiniti, mod pengikatan dan interaksi yang mungkin antara perencat dan PfGST. Dua tapak pengikatan telah dicadangkan untuk Hemin dalam PfGST. Bagaimanapun CB, GSX, EA dan HEPES tidak mampu bersaing dengan Hemin untuk mengeluarkannya daripada tapak pengikatannya dan menjadikan mereka tidak sesuai untuk digunakan sebagai petunjuk. Oleh kerana afiniti yang tinggi dan interaksi yang mungkin antara CB dan Hemin, struktur kristal kompleks CB dengan PfGST dikaji menggunakan X-ray kristalografi. Sebagai satu pendekatan alternatif untuk mendapatkan petunjuk, molekul telah direka untuk menyasarkan tapak pengikat
MOLECULAR CLONING, EXPRESSION, AND CHARACTERIZATION OF GLUTATHIONE-S-TRANSFERASE AS A NOVEL TARGET IN ANTI-MALARIAL DRUG DESIGN AND DISCOVERY

ABSTRACT

The Glutathione-S-transferases (GSTs) are group of detoxification enzymes. *Plasmodium falciparum* has a single isoform of GST (*Pf*GST) that involves in heme detoxification. While other GSTs isoforms from human (hGSTP1) and mouse (mGSTM1) are involved in apoptotic stress kinase pathway and mediate cancer cell resistance to chemotherapy. The *Pf*GST, hGSTP1 and mGSTM1 were successfully cloned and heterologously expressed in *E. coli*. Enzyme inhibition, kinetics and crystallization experiments were conducted to find potential lead compounds that inhibit *Pf*GST. The GSTs substrates, glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB), as well as the known GSTs inhibitors of S-hexyl glutathione (GSX), cibacron blue (CB), ethacrynic acid (EA), hemin, protoporphyrin IX (protoIX) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were studied. The results revealed affinities, binding modes and possible interactions between the inhibitors and *Pf*GST. Two binding sites were proposed for hemin in *Pf*GST. However, CB, GSX, EA and HEPES were unable to compete with hemin binding, thus considered unsuitable leads to dislodge hemin from its binding site. Due to its high affinity and possible interaction with hemin, the crystal structure of CB in complex with *Pf*GST was studied further using X-ray crystallography. As an alternative approach to obtain leads, molecules were computationally designed for targeting GSH binding site in *Pf*GST and destabilize hemin binding. Leads based on GSH molecule
and/or its binding pocket were searched using de novo approach. Three approaches were developed for reversible inhibitors using fragment-based and atom-based approaches, and one approach for irreversible inhibitors design. The isosteric-fragment replacement (IFR) and docked-fragment replacement (DFR) approaches successfully generated de novo ligands with free energy of binding and synthetic accessibility score were calculated for lead selection. Molecular dynamic simulation for selected IFR and DFR ligands showed that 1598-DFR maintain stable binding with free energy of binding of -16 kcal/mol and RMSD of less than 3 Å throughout simulation period of 7.5 ns. The third approach generated lead molecules by atomic assembly using Structure-Assisted Atom-based De novo molecular design (SAAD) which tailor made the molecular fragments to fit the binding pocket of γ-glutamyl moiety of GSH. In the last approach, irreversible specific inhibitors were designed to form covalent bond with PfGST by using its unique 3-dimensional arrangement of cysteine residues. The analogues were designed to establish covalent bond with Cys101 through electrophilic moieties that replaces α-amino of the γ-glutamyl of GSH. The designed lead molecules may open a new avenue for treating malaria by reversible and irreversible inhibition of PfGST. Plant extracts were screened as an alternative source for lead compounds. The highest PfGST inhibition was obtained using Cinnamomum iners bark, Terminalia catappa leaves and Phyllanthus watsonii leaves. The kinetic results suggest that these plants inhibited PfGST via competing with CDNB.
CHAPTER 1
INTRODUCTION

1.1 Problem statement

The discovery of new class of anti-malarial compounds is highly recommended specially after development of resistant strains for *Plasmodium falciparum*. The resistance is developed by mutations at enzymes or transmembranal transporters which are interacted with anti-malarial compounds. Such mutations are usually occurred close to the active site of those macromolecules and consequently disrupt their proper functions. However, the harmful biochemical consequences of such disruption can be relieved if the genome encodes other isoforms of the mutated macromolecule.

Glutathione-S-transferases (GSTs) are group of detoxification enzymes that conjugate xenobiotics or hydrophobic molecules to endogenous substrate of glutathione (GSH). The conjugation product is more polar and thus suitable for subsequent elimination from the cell (Hinchman and Ballatori, 1994). Moreover, GSTs have been reported to catalyze isomerization reactions as well as involve in small molecular carriage and protein interactions (Oakley, 2011).

The *Plasmodium falciparum* genome encodes single isoform of GST (*Pf*GST) which is being involved in heme capturing (Harwaldt et al., 2002; Deponte and Becker, 2005; Hiller et al., 2006). Thus *Pf*GST provides a potential target for anti-malarial drug discovery and development which could act synergistically with quinines (Harwaldt et al., 2002). The structural differences between *Pf*GST and human GSTs can be involved to promote selectivity during drug design and screening (Fritz-Wolf et al., 2003).
Other GST isoforms like human Pi-1 (hGSTP1) and mouse Mu-1 (mGSTM1) have been reported to involve in apoptotic stress kinase pathway. The apoptosis signal regulating kinase-1 and its substrate C-Jun N-terminal kinase (JNK) are inactivated by interaction with mGSTM1 (Cho et al., 2001) and hGSTP1 (Adler et al., 1999), respectively. Moreover, the overexpression of hGSTP1 in malignant cells was found to promote resistance toward alkylating agents (Parker et al., 2008). Therefore, ethacrynic acid (a GST substrate and inhibitor) has been used in conjugation with chemotherapies to potentiate cytotoxicity (Tew, 1994).

Cloning of cDNAs and heterologous expression of PfGST (Liebau et al., 2002), hGSTP1 (Moscow et al., 1989) and mGSTM1 (Townsend et al., 1989) have been previously conducted in E.coli using Plasmodium falciparum parasite, human lymphoblast, and mouse fibroblast as RNA sources, respectively. Biochemical and kinetic experiments have been conducted to study substrates and inhibitor binding to PfGST, hGSTP1 and mGSTM1. In case of limited X-ray crystallographic data, molecular docking provides a computational aid to interpret the observed kinetic behavior for those enzymes.

Plants are considered the origin of known anti-malarial like quinine (Lee, 2002b) and artemisinin (Lee, 2002a), as well as provide diverse library for hit screening (Harvey, 2007; Guantai and Chibale, 2011). Measuring the inhibitory effect of plant extracts toward PfGST, hGSTP1, and mGSTM1 has potential application for lead discovery as well as usage in malaria and cancer treatment.

Computational tools are currently being incorporated in molecular lead discovery and design. Depending on macromolecular crystal structure, high affinity molecules can be designed to fit the active site using de novo fragment-based or atom-based assembly. The stability and binding energy of the designed molecule at the
binding site can be tested by molecular dynamics simulation. With respect to the development of GSTs inhibitors, computational tools have seldomly being used with the exception of using combination of GRID and docking approaches to design simple GS-R derivatives (Procopio et al., 2005). Up to date, no isosteric replacements of GSH residues have been used to fully design GST inhibitors, with the exception of replacing Gly residue by tetrazole carboxylate isostere (Burg et al., 2002b) and γ-glutamyl-cysteine peptide bond with urethanic junction (Cacciatore et al., 2005). Yet, both replacements have been reported to adversely affect the activity of the prepared analogues. Several non-specific inhibitors have been developed by seeding GSH or other scaffolds (Mahajan and Atkins, 2005; Ruzza et al., 2009).

Since its discovery (Liebau et al., 2002), PfGST has not been the subject for specific lead discovery and design despite being frequently reported as valuable anti-malarial target (Srivastava et al., 1999; Harwaldt et al., 2002; Fritz-Wolf et al., 2003; Deponte and Becker, 2005). Which could be an application of neglectation for a member of bottom billion problems, where support is given for financial opportunities over global health needs (Trouiller et al., 2001). Therefore, the aim of this study is to clone and heterologously express GST isoforms of PfGST, hGSTP1 and mGSTM1. Enzyme kinetics and molecular docking are to be used for investigating the interaction between the GST isoforms and a group of GST inhibitors; which may shape and address potential leads. X-ray crystallography is to be used to resolve the binding mode of PfGST inhibitor. Several plant extracts and fractions are to be screened for comparative inhibitory effect toward the GSTs isoforms. Computational tools are to be involved in lead molecules design and validation. Atom-based and fragment-based de novo molecular design approaches are to be developed and applied for PfGST inhibitor design.
1.2 Malaria and anti-malarial compounds

Malaria is one of the most devastating endemically reemerging protozoal disease which is no more restricted to poor and developing countries. According to reports in 2014 from World Health Organization and Global Malaria Action Plan, malaria infects 198 million people and kills about 600,000 annually. And according to the reports, about half of world population is at risk of being infected with malaria and there are 79 malarious countries of which 20 countries are in Asia-Pacific (Figure 1.1). In spite of global spread of malaria and development of drug resistant strains, no new chemical class of anti-malarial has been introduced to clinical practice since 1996 (Gamo et al., 2010).

Malaria causative agent is the *Plasmodium* parasite. Until now, more than 100 species of *Plasmodium* have been identified (Tuteja, 2007), however at least 5 species are currently known to infect human with the most virulent being *Plasmodium falciparum* (Kantele and Jokiranta, 2011). The parasite multiplies sexually in the gut of *Anapheles* mosquito to form sporozoites which migrate to insect’s saliva and is injected to host blood stream when the insect takes its meal. Subsequently, the sporozoites reach and multiply asexually in host’s liver (5-15 days) before shizonts rupture and release merozoites. Merozoites infect red blood cells and occasionally form the sexually mature gametes; the forms which are suitable for multiplication in insect gut (Laurence et al., 2008).
Figure 1.1: Global distribution for percentage of malaria cases caused by Plasmodium falciparum (WHO mapper, 2014).
During the intra-erythrocytic stage, the parasite digests cellular haemoglobin of the host to get the necessary amino acids by using several proteinases. The unavoidable consequence of haemoglobin digestion is the release of free ferroprotoporphyrin. In the presence of oxygen ferroprotoporphyrin (heme) is oxidized to ferriprotoporphyrin (hemin). This process produces superoxide, which decomposes into $H_2O_2$ and $O_2$. The parasite detoxify free heme by crystallizing it into hemozoin (Egan et al., 2002). Anti-malarial of 4-aminoquinolines like chloroquine and amodiaquine inhibit heme accumulation into hemozoin, thus building up a toxic concentration of ferro/ferriprotoporphyrin leading to parasite death (Deharo et al., 2003).

At the host side, human body reacts against infection by production of nitrous oxide and oxygen radicals. Host reaction as well as parasitic digestion of haemoglobin and formation of free heme exaggerates the oxidative stress in the parasite. The parasite relieves the oxidative stress via glutathione and thioredoxin dependent enzymes systems (Krnajski et al., 2002; Becker et al., 2004).

Several anti-malarial compounds are available (Figure 1.2) and act at different stages through the life cycle of the parasite (Figure 1.3). Although the molecular targets have not been determined for many of the compounds, the currently known targeted pathways are mainly related to nucleic acid metabolism, heme detoxification, oxidative stress, protein digestion, fatty acid biosynthesis, and trans-membranal channels (Alam et al., 2009).

Resistance toward anti-malarial compounds is attributed to mutation in key enzymes or in compounds trans-membranal transporters. For example, resistance toward chloroquine is attributed to mutation in Pfmdrl, Pfeg2 and Pfcr1 transporters, atovaquone to mutation in cytochrome b, anti-folates (pyrimethamine and proguanil)
to mutations in dihydrofolate reductase, sulfonamides and sulphones to mutations in dihydropteroate synthase, and even artemisinin due to mutations in PfATPase6 (Olliaro, 2001; Alam et al., 2009).

The *Plasmodium falciparum* (isolate 3D7) has 23-megabase nuclear genome consists of 14 chromosomes, encodes about 5,300 genes, and is the most (A + T)-rich genome sequenced to date (Gardner et al., 2002a). Enzymes form about 15% of the predicted expressed proteins (Figure 1.4) and considered as anti-malarial targets (Table 1.1).
Figure 1.2: The main classes of anti-malarials. The chemical structures of all the main classes of anti-malarials and other therapeutic and control molecules are assembled according to either the chemical classes they belong to (endoperoxides, 4- and 8- AQs, amino-alcohols) or their function (antifolate, antibiotics), or both (e.g., sulfonamides, a chemical class of antibiotic used in combined anti-malarial therapies) (Delves et al., 2012).
Figure 1.3: Summary of the activity of the most widely used anti-malarials throughout the life cycle of *Plasmodium*. The three main phases, i.e., liver stage, blood stage, and vector stage, of the life cycle of *Plasmodium* are shown. The two key entry points leading to transmission of the parasites from vector to host and from host to vector are indicated (green circles). Parasite forms specific to each stage are highlighted and drugs identified as inhibitors of development of these forms are listed in boxes and colored as described in previous Figure. Stars highlight components of the main artemisinin combination therapies: green, coartem; red, pyramax; orange, eurartesim; blue, ASAQ (Delves et al., 2012).
Figure 1.4: Classification of functional proteins predicted from *Plasmodium falciparum* (3D7) genome (Gardner et al., 2002a).

Table 1.1: Enzymes considered as anti-malarial targets (Mehlin, 2005; Buchholz et al., 2007; Alam et al., 2009).

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidases</td>
<td>Plasmepsin</td>
</tr>
<tr>
<td></td>
<td>Faclipain</td>
</tr>
<tr>
<td></td>
<td>Aminopeptidase N falcilysin</td>
</tr>
<tr>
<td>Glycolytic enzymes</td>
<td>L-lactate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Triose phosphate Isomerase</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Fructose-1,6-bisphosphate aldolase</td>
</tr>
<tr>
<td>Lipid metabolizing enzymes</td>
<td>Enoyl-acyl carrier reductase</td>
</tr>
<tr>
<td></td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>Redox and detoxification enzymes</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td></td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>Folate synthesis</td>
<td>Dihydrofolate reductase-thymidylate synthase</td>
</tr>
<tr>
<td>Purine salvage enzymes</td>
<td>Purine Nucleoside Phosphorylase</td>
</tr>
<tr>
<td></td>
<td>Adenylosuccinate synthetase</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine phosphoribosyltransferase</td>
</tr>
</tbody>
</table>
1.3 Glutathione-s-transferases (GSTs)

Glutathione-S-transferases (GSTs, E.C. 2.5.1.18) are ubiquitous family of enzymes involved preliminarily in nucleophilic substitution reactions. GSTs can bind to endogenous as well as exogenous substrates. Exogenous substrates represent drugs, industrial intermediates, pesticides, herbicides, environmental pollutants, and carcinogens. While endogenous substrates represent cell-membrane phospho-lipid hydroperoxides, oxidized products of nucleotides and catecholamines, as well as several other endogenous compounds.

GSTs were first studied as xenobiotic metabolizers before other functions were reported. Back to 1960s, the rate liver extracts showed the ability to catalyze the conjugation of 1,2-dichloro-4-nitrobenzene to glutathione tripeptide (γ-glutamyl-cysteinyl-glycine, GSH) (Figure 1.5). Other activities such as binding carcinogens, steroids and bilirubin were also reported, thus the protein was initially called ‘ligandin’. The previous activities were –later on- attributed to Mu and Alpha classes of GSTs. Several classes of GSTs were discovered by using 1-chloro-2,4-dinitrobenzene (CDNB) as a more general transferase substrate as well as using bioinformatics approach (Sherratt and Hayes, 2002).

GSTs belong to phase-II detoxification enzymes that remove electrophilic compounds from the cell by conjugation to endogenous GSH. The GS-conjugates are substrates for transmembranal transporters. GSTs protect the cell from environmental and oxidative stress, xenobiotics, as well as responsible for resistance against certain drugs, therefore, have been considered a reliable target in several therapeutic designs and interventions. In this section a biological introduction will be given regarding classification, structure, functions, and medical significance of GSTs. Members of GSTs family from *Plasmodium falciparum*, human, and mouse will be considered.
Figure 1.5: Structure of tripeptide glutathione (γ-glutamyl-cysteinyl-glycine or GSH)

1.4 Classification of GSTs

Within creatures, three classes of proteins so far have been discovered that show glutathione transferase activity. Two of them are soluble, namely cytosolic and mitochondrial GSTs. The third is membrane-bound microsomal GST also referred as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). Variations in structure and substrate selectivity represent the main differences between soluble and membrane-bound GSTs. Soluble forms are more directed toward drugs and xenobiotics metabolism while membrane-bound toward leukotrienes and prostaglandins metabolism. All soluble and membrane-bound GSTs can conjugate 1-chloro-2,4-dinitrobenzene to GSH and exhibit glutathione-dependent peroxidase activity toward cumene hydroperoxides (Holm et al., 2002; Hayes et al., 2005; Holm et al., 2006).

About 15-20 different cytosolic GSTs have been identified in human and mammals, 40-60 in plants, 10-15 in bacteria, and more than 10 in insects (Nebert and Vasiliou, 2004; Frova, 2006). Cytosolic GSTs can be classified into 16 classes depending on sequence similarity, immunological cross reactivity, and substrate specificity, namely: alpha, beta, delta, epsilon, zeta, theta, kappa, lambda, mu, nu, pi, sigma, tau, phi, omega and dehydroascorbate reductase (DHAR) (Mannervik and
Danielson, 1988; Salinas and Wong, 1999; Strange et al., 2000; Strange et al., 2001; Frova, 2006; Oakley, 2011). Some subclasses are ubiquitous, others are organism-specific. Currently recognized classes of cytosolic GSTs in mammals include alpha, mu, omega, pi, sigma, theta and zeta (Mannervik et al., 2005). GSTs show higher interclass sequence similarity (60-80%) than structural homology (25-35%) (Andujar-Sanchez et al., 2005).

### 1.5 General structure of cytosolic GSTs

Microsomal GSTs or Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEGs) are present as homotrimeric membrane-spanning helical structures (Holm et al., 2006). On the other hand, soluble GSTs are all functional homo or hetero (within same class) dimeric. Each monomer is of 199–244 amino acids in length and has molecular weight from 23-28 kDa (Armstrong, 1997). It is composed of a conserved N-terminal thioredoxin domain containing GSH binding site connected to a more variable C-terminal R-helical domain containing the binding site for the GSH acceptor substrate (Hayes et al., 2005) (Figure 1.6).

The typical thioredoxin domain composed of β-α-β-α-β-β-α structural motif (Armstrong, 1997; Hebert and Jegerschold, 2007; Atkinson and Babbitt, 2009) (Figure 1.7). The active site within the thioredoxin domain which recognizes GSH (G site) is conserved in all classes of GSTs, however, some residues may vary among different classes. The residue of major interest in the binding site is the one which principally activates the thiol group of GSH for nucleophilic attack (Armstrong, 1997). For example, the binding site of alpha, mu, and pi classes in mammals as well as PfGST utilizes tyrosine residue for GSH activation. The classes of phi and tau in plants, delta in insects as well as theta and zeta utilize serine residue. While omega in
mammals and insects, beta in bacteria, lamda and DHAR in plants utilize cysteine residue (Frova, 2006). Due to the formation of mixed disulfide bond with GSH, omega class of GSTs has poor conjugation activity, instead; it is involved mainly in redox reactions (Whitbread et al., 2005). In all of alpha, mu, and pi classes, the GSH tripeptide adopts an extended conformation running antiparallel to the conserved loop (50-53) that connects α2 and β3 (Fritz-Wolf et al., 2003). The α-amino group of γ-Glu interacts with strictly conserved Gln and Asp residues. However, the fundamental interaction found in all classes of cytosolic GSTs is the hydrogen bonding between Pro residue at the N-terminus of β3 and backbone amine of GSH cysteiny moiety (Oakley, 2011).

The second domain contains the hydrophobic substrate binding site (H-site) and consists of a variable number (4–7) of α-helices positioned downstream the thioredoxin domain and connected to it via short loop (around 10 amino acids). GSTs sequence alignments from different species showed that about one third of the sequence differences are focalized at the H-site, thus determines the range and selectivity of chemicals metabolized by the enzyme (Armstrong, 1997). The G- and H-sites are labeled in Figure 1.11.
Figure 1.6: Topology and structural representations of (a) cytosolic GST, (b) mitochondrial GST, and (c) MAPEG. The thioredoxin domain (green) composed from α-helices (circles) and β-sheets (triangles). The C-terminal domain composed mainly from α-helices (Oakley, 2011).

Figure 1.7: Thioredoxin domain common in soluble GSTs; it is composed from 4 beta-sheets interconnected by 3 alpha helices. The GSH binding to GST is stabilized by hydrogen bonds (dashed blue line). The GSH thiol group is activated by giving hydrogen bond to catalytic residue Tyr (Atkinson and Babbitt, 2009).
1.6 General mechanisms for catalyzing chemical reactions employed by enzymes and GSTs

Enzymes are merely catalysts for chemical reactions, they only accelerate rate of chemical reaction toward the equilibrium by lowering activation energy and not changing the equilibrium. Enzymes (E) induced strains and perturbations that convert substrate (S) to its highly energized unstable transition state structure (ES‡). The short half-life of transition state (about $10^{-13}$ second) is extended by binding enzymes, thus reduces the required activation energy ($\Delta G_{ES^\dagger}$). According to Arrhenius equation, linear lowering of activation energy separating for a given reaction, produces exponential increment in reaction rate (Equation 1.1).

$$\text{Reaction rate constant} = Ae^{-\frac{\Delta G_{ES^\dagger}}{RT}}$$  \quad \text{Equation 1.1}

Where R is the gas constant, T is the temperature in Kelvin, and A is the Arrhenius constant. Several mechanisms have been proposed for ES‡ complex stabilization (Copeland, 2000):

**Structural features of enzyme active site:**

- Sequester the substrate from solvent effect.
- Decrease dielectric constant by the hydrophobic pocket, thus, intensifying the electric field against the substrate produced by judiciously placed charged functional groups within the active site.

**Reactivity features of enzyme active site:**

- Approximation of reactants and orbital steering
- Covalent catalysis (nucleophilic and electrophilic addition)
- General acid-base catalysis
- Conformational distortion
- Preorganization of the active site for transition state complementarity

GST group of enzymes catalyzes the conjugation reaction of GSH by nucleophilic addition. The enzyme active site involves in activating the thiol group of GSH to form partial covalent bond between the tyrosine or serine residue and thiolate ion of GSH. Subsequently, the activated thiolate ion can easily attack any electrophilic group of the second substrate (Graminski et al., 1989; Shan and Armstrong, 1994; Armstrong, 1997).

Generally in nucleophilic catalysis, the reaction rate depends on nucleophilic strength and electron donating ability of the attacking group which in turn directly related to basicity (pKa). Moreover, it depends on the group’s oxidation potential, polarizability, ionization potential, electronegativity, potential energy of the higher occupied molecular orbital (HOMO), covalent bond strength, and general size of the group.

With respect to substrate, the reaction rate depends on the electrophilicity of the substrate functional group (i.e. how good its “leaving group” is), pKa of leaving group, hence, its state of protonation (the weaker is the base, the better is the leaving group species), and chemical nature of the leaving group (Copeland, 2000).

With respect to GSTs, the enzyme stabilizes the thiolate form of GSH, thus making it available for conjugation. All of P/GST, hGSTP1, and mGSTM1 have been designed to use tyrosine residue for stabilizing GSH thiolate ion via hydrogen bond (Figure 1.8). Moreover, intra molecular interaction with the free amino group of γ-
glutamyl moiety appears to play a crucial role in activating the thiol group in GSH (Adang et al., 1988).

Figure 1.8: Conjugation reactions catalyzed by rate GST Mu 1-1 (rGSTM1-1, PDB 5FWG) that uses Tyr6 for activating GSH thiol group. The active thiolate group attacks a) phentrene epoxide by nucleophilic addition and b) benzylideneacetone double bond by Michaelis addition (Shan and Armstrong, 1994).
1.7 GSTs functions and substrates

The main function of GSTs is to catalyze the conjugation of GSH toward xenobiotic substrates. However, other functions are vested to GSTs including peroxide degradation, double-bond cis-trans isomerization, steroid and leukotriene biosynthesis, reduction and non-catalytic “ligand-in” activities.

1.7.1 Conjugation activity

GST is defined as a member of phase-II detoxification enzyme. GSTs catalyze variety of reactions involving endogenous and exogenous compounds as substrates. Nucleophilic attack of GSH to nonpolar molecules which carry electrophilic C, N, or S atoms is the most important cellular defense reaction catalyzed by GSTs. In human, GSH conjugates are, subsequently, expelled from the cell by trans-membranal ATP-Binding Cassett (ABC) transporters. GSH conjugates are then metabolized by extracellular proteins γ-glutamyltransferase and dipeptidases to sequentially remove glutamyl and glycyl moieties, respectively. Specific cells reabsorb cysteine S-conjugates and perform acetylation on the amino group of the cysteinyl residue by intracellular N-acetyl-transferases. Corresponding mercapturic acids (N-acetylcysteine S-conjugates) is then formed that can be released into the circulation and delivered to the kidney for excretion in urine, or they may undergo further metabolism (Hinchman and Ballatori, 1994)

Substrates that can be detoxified by GSTs include cancer chemotherapeutic agents specially alkylating agents such as busulfan, carmustine, chlorambucil (Parker et al., 2008), cis-platin, cyclophosphamide, etoposide quinone (metabolite of Etoposide), ethacrynic acid, melphalan, mitozantrone, and thiotepa. Environmental chemicals and their metabolites also detoxified by GST include acrolein, atrazine,
DDT, inorganic arsenic, lindane, malathion, methyl parathion, muconaldehyde, and tridiphane. Another important class of substrates for conjugation by GSTs is the in vivo products of phase-I transformation, like phenanthrene epoxide, benzo[α]-pyrene epoxide, and lipid peroxides (Hayes et al., 2005). Other substrates include herbicides (Marrs, 1996; Neuefeind et al., 1997), pesticides, industrial intermediates, environmental pollutants, carcinogens, heterocyclic amines produced by cooking protein-rich food, arene oxides, unsaturated carbonyls, and organic halides (Hayes et al., 2005; Oakley, 2011). A list of conjugation reactions catalyzed by GSTs is provided in Figure 1.9.

1.7.2 Peroxidase activity

GSTs possess selenium-independent glutathione peroxidase activity towards organic hydroperoxides. This activity is cellular protective since it prevents organic hydroperoxides of phospholipids, fatty acids and DNA in becoming engaged in free radical propagation reactions which ultimately lead to macromolecular destruction (Hayes and Strange, 1995; Deponte and Becker, 2005). GSTs can detoxify products of lipid peroxidation (Bruns et al., 1999; Collinson et al., 2002), polycyclic aromatic hydrocarbon epoxides derived from the catalytic actions of phase 1 cytochrome P-450s as well as numerous by-products of oxidative stress (Strange et al., 2001).

1.7.3 Isomerization activity

Some GSTs isozymes are known to possess GSH-dependent isomerization activities, like isomerization of the keto steroid intermediates in testosterone and progesterone synthetic pathway (Johansson and Mannervik, 2001), the less hydrophilic maleate analogues of maleylacetoacetate (Keen and Jakoby, 1978; Fernandez-Canon and Penalva, 1998), and 13-cis retinoic acid (Chen and Juchau,
Some GST isoforms are involved in biosynthesis of arachidonic acid derivative of eicosanoids like prostaglandins and leukotrienes (Kanaoka et al., 1997). Some parasites are able to use this characteristic of GSTs to perform GSH-dependent isomerization of some chemicals such as prostaglandins, thus modulating the host immune system during infection (Angeli et al., 2001; Ouaissi et al., 2002).

1.7.4 Toxicity potentiation activity

Opposite to detoxification, GSTs can potentiate the toxicity of some substrates by catalyzing conjugation or lysis reactions. As example GSTs increases the toxicity of some short chain alkyl halids that have two functional groups like dihalomethane, dihaloethane, isoesters, isothiocyanates, sulforaphane, and haloalkene. Moreover, GSTs can release cytotoxic drugs from prodrug contains sulfide bond such as the conversion of azathioprine to mercaptopurine (Eklund et al., 2006). This phenomenon has been efficiently incorporated in designing tumor selective chemotherapy where the overexpressed GSTs activate the release of active cytotoxic molecular fragment (Lyttle et al., 1994b; Satyam et al., 1996; Rosen et al., 2003; Ruzza and Calderan, 2013). Another example is the TER 286 which can be activated by the overexpressed GST (hGSTP1) in cancerous cells to generate nitrogen mustard alkylating agent (Morgan et al., 1998).

1.7.5 Ligand-in activity

GSTs can function as cargo proteins to carry certain organic molecules by non-productive ligand-in process (Mannervik and Danielson, 1988). Ligand-in is another mechanism to detoxify compounds under condition of lower GSH concentration (Parker et al., 2008). GSTs can carry hemin, bilirubin, bile salts, steroids (Remoue et al., 2002), thyroid hormones, fatty acids (Caccuri et al., 1990). Moreover, GSTs can
detoxify several drugs by ligand-in like adriamycin, bleomycin, mitomycin C, carboplatin (Ruzza et al., 2009), as well as the anti-inflammatory drug sulfasalazine (Oakley et al., 1999). The process of ligand-in is applied for hemin transportation (Boyer and Olsen, 1991) giving hematin binding in *Haemonchus contortus* as an example of endowment for parasite to tolerate blood feeding (van Rossum et al., 2004). Moreover, it was recognized that GST in *Plasmodium falciparum* could function as a buffer for heme-containing compounds in vivo (Platel et al., 1999; Harwaldt et al., 2002; Deponte and Becker, 2005; Liebau et al., 2005).

### 1.7.6 Protein-interaction activity

Soluble GSTs can bind to some proteins and manipulate their functions. The protein kinases involved in apoptotic stress kinase pathway can be inactivated by interaction with some GST isoforms. The apoptosis signal regulating kinase-1 and its substrate C-Jun N-terminal kinase (JNK) are inactivated by interaction with mGSTM1 (Cho et al., 2001) and hGSTP1 (Adler et al., 1999), respectively. The oxidative or chemical stress induced by chemotherapies mediate the dissociation GST-JNK-C-Jun complex leading to apoptosis (Adler et al., 1999; Townsend, 2007).

Other functions of GSTs include reduction of dehydroascorbate and transfer of thiols (Neuefeind et al., 1997), dehydroascorbate reductase activities (Board et al., 2000), participation in cellular signaling, regulation of transcription and stress response (Salinas and Wong, 1999), as well as catalyzing formation of disulfide bonds for some hydrophobic substrates (Keen and Jakoby, 1978). Selected reactions catalyzed by GSTs are provided in Figure 1.10.
Figure 1.9: Conjugation reactions catalyzed by GST for a) 1-chloro-2,4-dinitrobenzene (CDNB), b) chlorambucil, c)sulforaphane, d) ethacrynic acid, e) benzylideneacetone, f) phenanthrene epoxide a product of cytochrome P450 catalyzed oxidation of phenanthrene.
Figure 1.10: Reactions catalyzed by GST; a) reduction for cumene, b) \( \sigma \)-dopaquinone conjugation, c,d) thiolysis for 4-nitrophenyl acetate and trinitroglycerin, f) isomerization of maleylacetoacetate, f) activation of 1,2-dibromoethane and g) conversion of PGH\(_2\) to PGD\(_2\).