Intl. J. Bioinformatics and Biological Sci., 4(2): 67-75, December 2016

DOI: 10.5958/2321-7111.2016.00009.3

A Presumed Checkpoint in the *P. falciparum* Malarial Infection

Pratim Chakraborti*, Pallavi Sharma, Soumita Paul and Aparna Karmakar

Apt Software Avenues Private Limited, Unit G301 & 302, Block DC, City Centre, Salt Lake, Kolkata, India

*Corresponding author: pratimc@aptsoftware.com

ABSTRACT

Malaria is a serious issue for its negative impact on the community and the increasing resistance to drugs. Hence the treatment of malaria is a great challenge for the community. While studying the complex interaction of molecules between host and the malarial parasite from the point of entry and the subsequent invasion within the RBC, it seemed that there might be a certain crucial juncture where the process can be intervened. We have focussed on these interaction pathways and various antagonistic drugs that act in these pathways to deal with malarial infection. CQ and Art are the frequently used drugs worldwide but, now-a-days the event of they becoming resistant have also been reported. Mutations in Digestive Vacuole Membrane Transporters make CQ ineffective whereas, the reason behind the Art resistant is the mutation in the K13 gene. The K13 gene product binds with the Nrf2, a transcriptional factor of anti-oxidants producing gene. This binding makes the Nrf2 ineffective causing the death of the parasites under stressed condition. Our aim of the study is to find a way out to inactivate Nrf2 in order to increase the Art efficiency.

Keywords: Malaria, Chloroquine, Artemisinin, PfPI3K, RBC

In human, malarial infection begins when an infected female anopheles mosquito bites the human host during which the malarial parasites (sporozoite stage) get transferred into the blood stream. The sporozoite enters the parenchymal cells of the liver in about 3-30 minutes[1][2], where they multiply asexually by binary fission, referred to as hepatic schizogony[3]. With the rupturing of the parenchymal cells, thousands of merozoites are released into the bloodstream[4][5]. Once in the bloodstream, they invade the erythrocytes or Red Blood Cells (RBCs) through a series of events. First, the merozoite-coat proteins interact with the RBC membrane proteins in a specific ligand-receptor fashion to form unique invasion pathways[6-8]. After that the host hemoglobin is digested to hematin, which is highly toxic to the parasite. So it is promptly polymerized to hemozoin, an insoluble brown coloured non-toxic pigment[9][10]. The parasite further grows into the schizont stage followed by merozoite stage in the RBC. When the RBC ultimately ruptures, the merozoites are released into the bloodstream to further infect other RBCs. The sexual development is thought to occur in schizonts by forming sexual rings that ultimately grow into gametocytes. After 10–12 days of development, mature male and female gametocytes are taken up by its primary host, the mosquito, during their blood meal within which sexual phase of the parasite life cycle is completed. In the infection pathway, there are some factors which have the potential to form important drug targets and it seems that if blocked or inhibited, they can lead to the suppression of the disease as a whole. But the efficacy of the system has to be subjected to further *in silico*, in vivo and in vitro study, of which we intend to pursue only the first one.

Hemoglobin Digestion

Hemoglobin digestion is an essential step for the intraerythrocytic development of the malarial parasite (Fig. 1). The primary reason for this digestion seems to be the requirement of amino acids for the protein synthesis of the parasite[11]. The digestion occurs in the digestive vacuole (DV) of parasites by certain proteases (Table 1)[12].

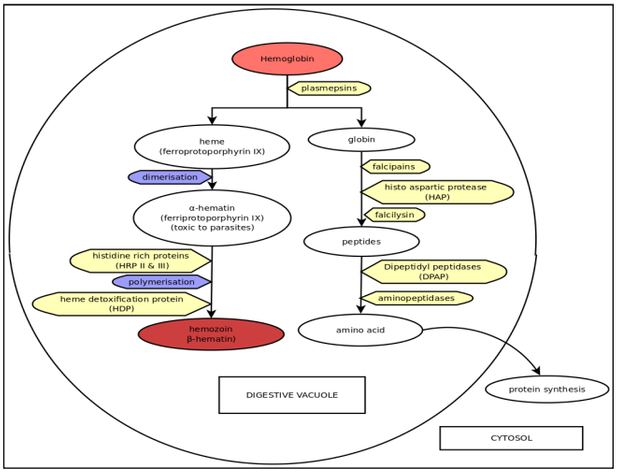

Fig. 1: Hemoglobin digestion in Digestive Vacuole

Table 1: Proteases involved in hemoglobin digestion.[12-25]

Proteases	Types	Protein ID (PlasmoDB)
Plasmepsin	Plasmepsin I	PF3D7_1407900
	Plasmepsin II	PF3D7_1408000
	Plasmepsin IV	PF3D7_1407800
Falcipain	Falcipain IIa	PF3D7_1115700
	Falcipain IIb	PF3D7_1115300
	Falcipain III	PF3D7_1115400
HAP	—	PF3D7_1408100
Falcilysin	—	PF3D7_1360800
DPAP	DPAP I	PF3D7_1116700
	DPAP II	PF3D7_1247800
Aminopeptidases	Leucyl aminopeptidase	PF3D7_1446200
	Aspartyl aminopeptidase	PF3D7_0932300
	Methionyl aminopeptidase Ia	PF3D7_0527300
	Methionyl aminopeptidase Ib	PF3D7_1015300
	Methionyl aminopeptidase Ic	PF3D7_ 0804400
	Methionyl aminopeptidase II	PF3D7_1434600
	AminopeptidaseP	PF3D7_1454400
	Subtilisin I	PF3D7_1136900
	Subtilisin II	PF3D7_0507500
	Subtilisin III	PF3D7_0507200
	Proline aminopeptidase	PF3D7_1401300
	Aminoacyl proline aminopeptidase	PF3D7_1454400
	Alanyl aminopeptidase	PF3D7_1311800

Upon hemoglobin digestion, heme is left behind. It is known to us that the heme (ferroprotoporphyrin IX) group gets dimerized into α-hematin (ferriprotoporphyrin IX (FP IX)) which is toxic to the parasites[24][26], as it causes peroxidative cleavage of unsaturated phospholipid in liposomes, leading to cell death[27]. So the parasitic proteases like heme detoxification protein (HDP) and histidine rich proteins (HRP II & III) polymerizes α-hematin to hemozoin[28][29]. Hemozoin, an insoluble crystalline brown coloured pigment (also known as β-hematin), is stored in the DV. These brown pigments play a major role in malarial diagnostic procedure[30].

Digestive Vacuole Membrane Transporters (DVMT) involved in antimalarial drug trafficking

Antimalarial drugs are administered to treat malarial infection. There are various transporters involved for the availability of these drugs to their respective targets. In normal eukaryotic cells, the xenobiotic toxicity is reduced by transporting the drug via specific transporters into the DV or lysosome from where they are further expelled out[31]. *Plasmodium* species being eukaryotic in nature have two types of transporters involved in the trafficking of xenobiotic compounds: P-glycoprotein (P-gh) related transporters and Drug Metabolite transporters (DMT)

system[32]. The P-glycoprotein related transporters or ABC transporters (ATP Binding Cassette transporters) influx the drug into the DVs from the cytosol whereas the DMT transporters efflux out the drugs from the DVs[32-34].

P-gh related transporters include many proteins of which *pfmdr1* (*Plasmodium falciparum* multidrug resistance-1) and *pfmrp* (*Plasmodium falciparum* multidrug resistance associated protein) caight our attention[35-37]. Both of them act as multidrug resistance proteins which remove drug from the cytosol by influxing it into the DV[35][36]. Both of them play important roles in the transportation of glutathione, chloroquine (CQ) and quinine (QN)[37].

The DMT system transporter include *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) protein which acts as an anion channel, effluxing out drugs like CQ from the DV[38][39]. Other than CQ, it also effluxes alkaloids, amine compounds, amino acids and peptides that are derived from the digestion of globins[40][41].

Chloroquine (CQ) : mode of action and its resistance

Chloroquine is the frequently used, as well as the most effective drug against malaria known till now[42]. When the CQ enters the DV, it becomes protonated due to the acidic environment of DV[43][44]. So it cannot leave the DV and thus interacts with the FP IX and forms FP IX-CQ complex in 2:1 stoichiometric ratio[45][46]. FP IX-CQ complex causes peroxidative cleavage of unsaturated phospholipid in liposomes in the same way as FP IX, but with higher efficiency[21]. This complex inhibits the conversion of hematin to hemozoin, causing toxicity to the parasite and finally leading to cell death[47].

According to records, DV membrane transporter proteins chiefly, *pfmdr1* and *pfmrp* promote the influx of CQ, but due to mutation the parasites became resistant to CQ (Fig. 2)[32]. There are many SNPs present all over the world for *pfmdr1,* among which, the frequency of N86Y is the highest, whereas no such records have been found for *pfmrp.* These mutations actively reduce the entry of CQ in DV[48-51].

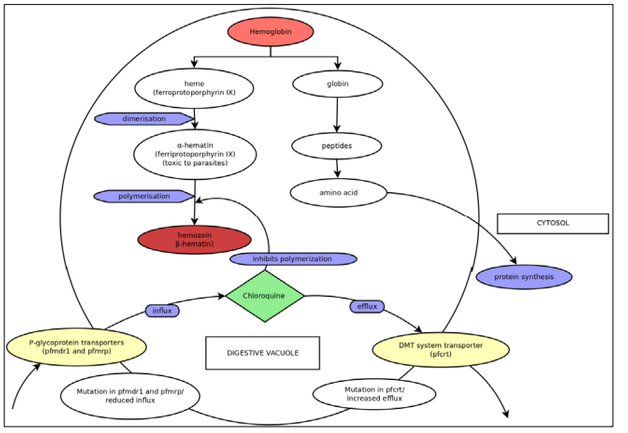

Fig. 2: Chloroquine mode of action and resistance

Mutation is also seen in *pfcrt*, which is responsible for efflux of the drug from DV. A number of mutations are seen in *pfcrt* of which K76T, Q271E, N326S, I356T, R371I are some of them. Out of these, K76T and R371I are most frequent[52]. These mutations increase the efflux of drug[38][53][54]. Mutation of *pfmdr1* and *pfcrt* does not allow the accumulation of sufficient amount of CQ in DV to act against parasites and thereby the effectiveness of CQ against malaria treatment has become questionable.

Artemisinin : mode of action and its resistance

The resistance of Malarial Parasites against CQ led to the urgent need for discovering novel antimalarial drugs. In the year 2011, a new drug named Artemisinin (Art) came to be known which was found to be effective against malarial parasite[55][56]. Art gets activated by Fe^{+2} ion of heme and releases 3 ROS (Reactive Oxygen Species) in DV[57-59]. Activated Art, also known as Dihydroartemisinin (DHA), alkylates the parasitic proteins in its cytosol. Alkylation causes disfunctioning of these proteins which leads to cell death[60-62]. To understand the Art resistance, it is important to know about the function of *Nrf2*, a transcriptional factor.

In eukaryotic cell, Nrf2 is free to bind with the promoter region of different anti-oxidant coding genes to assist in their transcription under stress condition (Fig. 3-B)[63][68][69]. Under no-stress, Nrf2

remains bounded to KEAP1 (product of K13 gene) for its ubiquitination through Cullin3 (Fig. 3-A). KEAP1 (product of K13 gene) acts as a substrate adapter for Cullin3 which is a ligase that ubiquitinates Nrf2, only if Nrf2 binds with KEAP1. As a result, no anti-oxidants are produced (Fig. 3-A)[71]. But under oxidative stress, ROS disrupts the critical cysteine residues in KEAP1. Due to this, Nrf2 gets detached from KEAP1 and thus Cullin3 cannot ubiquitinate Nrf2[72]. Now, this free Nrf2 moves into the nucleus and promotes the transcription of anti-oxidant producing genes (Fig. 3-B).

When Art is present in the cell, oxidative stress is increased due to the release of the 3 ROS at the time of Art activation (Fig. 3-C). This stress makes Nrf2 free to bind with the UDP-glucuronosyl transferases (UGT) coding gene assisting in its transcription[68] [69]. UGT (anti-oxidant) is responsible for production of glucuronic acid from DHA by glucuronidation. Glucuronic acid is subsequently effluxed out of the cell resulting in survival of the parasites (Fig. 3-D) [70]. But before this can occur, DHA alkylates all the parasitic protein causing death of the parasites (Fig. 3-E).

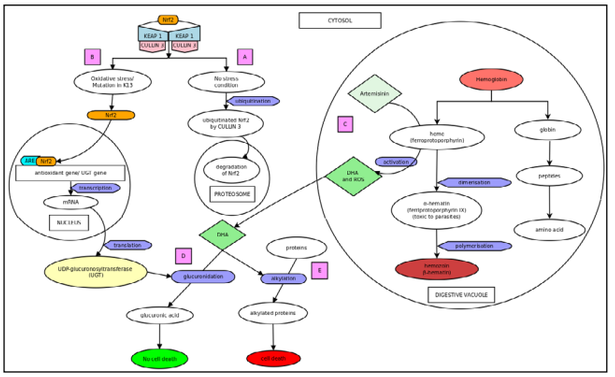

Fig. 3: Artemisinin mode of action and resistance

[A] No stress- Nrf2 degrades,
[B] Oxidative stress/ Mutation- Nrf2 promotes degradation of DHA,
[C] Activation of Artemisinin,
[D] Survival of the parasites due to DHA detoxification,
[E] Death of the parasites due to alkylation of the parasitic protein.

In case of Art resistance, UGT glucuronidates the DHA into glucuronic acid before it can alkylate the parasitic proteins[73]. This resistance occurs due to the several SNPs in K13 (Kelch like domain) gene which causes conformational changes in KEAP1. As a result, Nrf2 cannot bind with KEAP1, so it is free to produce UGT independent of the presence or absence of DHA[73]. Hence, Art is expelled out before it can act. Almost 15 SNPs are found all over the world, out of which three SNPs are seen to have higher frequency viz F446I (27.2%), P574L (6.7%) and C580Y (1.6%)[74].

RESULTS AND DISCUSSION

We have done an extensive review of a number of literatures pertaining to the life cycle of the parasite within the human host, specifically within the erythrocytes. The drugs which are administered to cure malaria are chloroquine (CQ) and very lately artemisinin (Art) has come into the scene. But individually both of them has turned somewhat ineffective, due to certain significant mutations that has occurred within the parasite genes. Hence the focus to cure malaria has shifted to a therapy known as ACT – Artemisin Combination Therapy[75]. But this too has its limitations[76]. There is a set of molecules, MSP or Merozoite Surface Protein, which are present in the parasites[77]. They bind with their receptor protein present on the host erythrocyte. This ligand-receptor interaction helps in the invasion of merozoites into the erythrocyte[6-8]. The unique binding sites of these proteins may act as a potential intervention point to inhibit the entry of merozoites into the erythrocytes. If merozoites are unable to enter into the erythrocytes, it will also be unable to produce malaria in the host. But there happens to be a number of MSPs on the surface of parasites that can help in invasion and it might be a mammoth task to identify the exact MSP which can be inhibited to restrict the entry of the parasite.

It is known that mutation in the drug transporter protein coding genes make CQ ineffective[52-54]. Also mutation in the K13 gene increase the Art resistance[73][74]. When Art enters into the parasitic DV, ROS is produced that increase the oxidative stress

of the cell (Fig. 3-C). Under oxidative stress, Nrf2, a transcription factor of anti-oxidant coding gene, does not bind with KEAP1, the K13 gene product. As a result of which, ubiquitination of Nrf2 is not possible by Cullin3 (that also binds with KEAP1) and Nrf2 produces anti-oxidant to reduce the stress, that leads to the survival of the parasites (Fig. 3-B, D). It is quite evident from studies that the production of anti-oxidant is slower than the alkylation of parasitic protein by activated Art (DHA)[73]. This leads to the death of the parasite due to this alkylation (Fig. 3-E). The mutation in the K13 gene, leads to a situation where Nrf2 cannot bind with the KEAP1-Cullin3 complex and produces anti-oxidants whether Art is present in the parasites or not (Fig. 3-A). Therefore Art becomes ineffective in those variants too. Such facts about the infection pathway enable us to understand that the binding of Nrf2 is important for the death of the parasites. This has turned our attention to a possible way out situation where blocking the Nrf2 with a proper inhibitor will cease the anti-oxidant production thereby increasing the effectiveness of Art and then the drug will presumably be able to play its desired role against the malarial infection. This way out we presume can enhance the efficiency of existing therapies. A similar but opposite outcome has been noticed in the case of proliferation of ovarian cancer cells[78]. Oxidative stress production increases in cancer cells and this stress signals help in the proliferation of cancer cells. So in this case, anti-oxidant production by Nrf2 is much essential to cease the proliferation of cancer cells. But in the case of malaria, the production of antioxidants will have to be reduced. This event strengthens our case, and we intend to embark upon more detailed analysis of our target, nrf2, and screening of its possible inhibitors.

CONCLUSION

An inhibitor that would attach to Nrf2 is a possible way to deal the malarial infection. We plan to study this transcription factor, Nrf2, to understand its primary structure and secondary structure in detail. We intend to carry out this study *in silico* in the beginning which might possibly encompass toxicity screening of the inhibitor and its derivatives, active site prediction of the target followed by prediction of binding energy of the inhibitor-target interaction. Thereafter we would plan for either *in vivo* or *in vitro* or *both* in order to validate our results.

REFERENCES

1. Gutierrez, Y. 2000. Apicomplexia of the Blood. In: Gutierrez, Y., editor. Diagnostic Pathology of Parsitic Infections with Clinical Correlations. 2nd Edition. New York, NY: Oxford University Press, pp. 235-262.
2. Goldberg, D.E., Slater, A.F.G., Cerami, A. and Henderson, G.B. 1990. Hemoglobin degradation in the malaria parasite Plasmodium falciparum: An ordered process in a unique organelle. *Proc. Natl. Acad. USA.* **87**: 2931–2935.
3. Goldberg, D.E., Slater, A.F., Beavis, R., Chait, B., Cerami, A., Henderson, G.B.1991. Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease. *J. Exp. Med.,* **173**: 961–969. [PubMed: 2007860]
4. Koch, M. and Baum, J. 2016. The mechanics of malaria parasite invasion of the human erythrocyte - towards a reassessment of the host cell contribution. *Cell Microbiol.* **18**(3): 319-29. doi: 10.1111/cmi.12557. Epub 2016 Jan 11. Review. PubMed PMID: 26663815; PubMed Central PMCID: PMC4819681.
5. Kauth, C.W., Epp, C., Bujard, H. and Lutz, R. 2003. The merozoite surface protein 1 complex of human malaria parasite *Plasmodium falciparum*: interactions and arrangements of subunits. *J Biol Chem.* **20**; 278(25): 22257-64. Epub 2003 Mar 24. PubMed PMID: 12654909.
6. Baldwin, M.R., Li X, Hanada T, Liu SC, Chishti AH. Merozoite surface protein 1 recognition of host glycophorin A mediates malaria parasite invasion of red blood cells. Blood. 2015 Apr 23;125(17):2704-11. doi: 10.1182/blood-2014-11-611707. Epub 2015 Mar 16. PubMed PMID: 25778531; PubMed Central PMCID: PMC4408295.
7. Sullivan DJ Jr, Gluzman IY, Goldberg DE. *Plasmodium hemozoin* formation mediated by histidine- rich proteins. Science. 1996; 271:219–222. [PubMed: 8539625]
8. Francis SE, Sullivan DJ Jr, Goldberg DE. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum. Annu. Rev. Microbiol.* 1997; 51:97–123. [PubMed: 9343345]
9. Sherman, I.W. 1977 . Amino acid metabolism and protein syn- thesis in malarial parasites. *Bull. WHO* 55:265 .
10. Goldberg DE, Slater AF, Beavis R, Chait B, Cerami A, Henderson GB. Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic

pathway initiated by a specific aspartic protease. *J Exp Med.* 1991 Apr 1;173(4):961-9. PubMed PMID: 2007860; PubMed Central PMCID: PMC2190804.

11. Hof F, Schütz A, Fäh C, Meyer S, Bur D, Liu J, Goldberg DE, Diederich F. Starving the malaria parasite: inhibitors active against the aspartic proteases plasmepsins I, II, and IV. *Angew Chem Int Ed Engl*. 2006 Mar 20;45(13):2138-41. PubMed PMID: 16502446.
12. P. S. Sijwali and P. J. Rosenthal, "Gene disruption confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 13, pp. 4384–4389, 2004.
13. P. S. Sijwali, J. Koo, N. Singh, and P. J. Rosenthal, "Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*," *Molecular and Biochemical Parasitology*, vol. 150, no. 1, pp. 96–106, 2006.
14. Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci U S A.* 2002 Jan 22;99(2):990-5. Epub 2002 Jan 8. PubMed PMID: 11782538; PubMed Central PMCID: PMC117418.
15. Murata CE, Goldberg DE. *Plasmodium falciparum* falcilysin: a metalloprotease with dual specificity. *J Biol Chem.* 2003 Sep 26;278(39):38022-8. Epub 2003 Jul 22. PubMed PMID: 12876284.
16. Tanaka TQ, Deu E, Molina-Cruz A, Ashburne MJ, Ali O, Suri A, Kortagere S, Bogyo M, Williamson KC. Plasmodium dipeptidyl aminopeptidases as malaria transmission-blocking drug targets. *Antimicrob Agents Chemother.* 2013 Oct; 57(10): 4645-52. doi: 10.1128/AAC.02495-12. Epub 2013 Jul 8. PubMed PMID: 23836185; PubMed Central PMCID: PMC3811399.
17. Rosenthal PJ. Falcipains and other cysteine proteases of malaria parasites. *Adv Exp Med Biol.* 2011; 712:30-48. doi: 10.1007/978-1-4419-8414-2_3. Review. PubMed PMID: 21660657.
18. Gardiner DL, Trenholme KR, Skinner-Adams TS, Stack CM, Dalton JP. Overexpression of leucyl aminopeptidase in *Plasmodium falciparum* parasites. Target for the antimalarial activity of bestatin. *J Biol Chem.* 2006 Jan 20;281(3):1741-5. PubMed PMID: 16286469.
19. Dalal S, Klemba M. Roles for two aminopeptidases in vacuolar hemoglobin catabolism in *Plasmodium falciparum*. *J Biol Chem*. 2007 Dec 7;282(49):35978-87. PubMed PMID: 17895246.
20. Zhang P, Nicholson DE, Bujnicki JM, Su X, Brendle JJ, Ferdig M, Kyle DE, Milhous WK, Chiang PK. Angiogenesis inhibitors specific for methionine aminopeptidase 2 as drugs for malaria and leishmaniasis. *J Biomed Sci.* 2002 Jan-Feb;9(1):34-40. PubMed PMID: 11810023.
21. Blackman MJ, Fujioka H, Stafford WH, Sajid M, Clough B, Fleck SL, Aikawa M, Grainger M, Hackett F. A subtilisin-like protein in secretory organelles of *Plasmodium falciparum* merozoites. *J Biol Chem.* 1998 Sep 4;273(36):23398-409. PubMed PMID: 9722575.
22. da Silva FL, Dixon MW, Stack CM, Teuscher F, Taran E, Jones MK, Lovas E, Tilley L, Brown CL, Trenholme KR, Dalton JP, Gardiner DL, Skinner-Adams TS. A *Plasmodium falciparum* S33 proline aminopeptidase is associated with changes in erythrocyte deformability. *Exp Parasitol.* 2016 Oct;169:13-21. Doi: 10.1016/j.exppara.2016.06.013. PubMed PMID: 27373432.
23. Skinner-Adams TS, Stack CM, Trenholme KR, Brown CL, Grembecka J, Lowther J, Mucha A, Drag M, Kafarski P, McGowan S, Whisstock JC, Gardiner DL, Dalton JP. *Plasmodium falciparum* neutral aminopeptidases: new targets for anti-malarials. *Trends Biochem Sci.* 2010 Jan;35(1):53-61. doi: 10.1016/j.tibs.2009.08.004. Review. PubMed PMID: 19796954.
24. Chugh M, Sundararaman V, Kumar S, Reddy VS, Siddiqui WA, Stuart KD, Malhotra P. Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*. *Proc Natl Acad Sci U S A.* 2013; 110:5392–5397. [PubMed: 23471987]
25. PlasmoDB.org
26. Orjih AU. On the mechanism of hemozoin production in malaria parasites: activated erythrocyte membranes promote beta-hematin synthesis. *Exp Biol Med (Maywood).* 2001; 226:746–752. [PubMed: 11520940]
27. Sugioka Y, Suzuki M, Sugioka K, Nakano M. A ferriprotoporphyrin IX-chloroquine complex promotes membrane phospholipid peroxidation. A possible mechanism for antimalarial action. *FEBS Lett.* 1987 Nov 2;223(2):251-4. PubMed PMID: 3666151.
28. Jani D, Nagarkatti R, Beatty W, Angel R, Slebodnick C, Andersen J, Kumar S, Rathore D. HDP-a novel heme detoxification protein from the malaria parasite. PLoS Pathog. 2008; 4:e1000053. [PubMed: 18437218]
29. Sullivan DJ Jr, Gluzman IY, Goldberg DE. *Plasmodium hemozoin* formation mediated by histidine-rich proteins. *Science.* 1996; 271:219–222. [PubMed: 8539625]
30. Yao MX, Wang H. [Hemozoin detection in malaria diagnosis]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2014 Feb;32(1):68-71. Review. Chinese. PubMed PMID: 24822371.
31. P.D. Dobson, K. Lanthaler, S.G. Oliver, and D.B. Kell, "Implications of the dominant role of transporters in drug uptake by cells," *Current Topics in Medicinal Chemistry*, vol. 9, no. 2, pp. 163–181, 2009.

32. T.J.C. Anderson, S. Nair, H. Qin *et al.*, "Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance?" *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 6, pp. 2180–2188, 2005.
33. A.D. Djimdé, O.K. Doumbo, J.F. Cortese *et al.*, "A molecular marker for chloroquine-resistant falciparum malaria," *The New England Journal of Medicine*, vol. 344, no. 4, pp. 257–263, 2001.
34. K.L. Waller, R.A. Muhle, L.M. Ursos *et al.*, "Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter," *Journal of Biological Chemistry*, vol. 278, no. 35, pp. 33593–33601, 2003.
35. M.T. Duraisingh and A.F. Cowman, "Contribution of the pfmdr1 gene to antimalarial drug-resistance," *Acta Tropica*, vol. 94, no. 3, pp. 181–190, 2005.
36. P. Rohrbach, C. P. Sanchez, K. Hayton *et al.*, "Genetic linkage of pfmdr1 with food vacuolar solute import in *Plasmodium falciparum*," *The EMBO Journal*, vol. 25, no. 13, pp. 3000–3011, 2006.
37. D.K. Raj, J. Mu, H. Jiang *et al.*, "Disruption of a *Plasmodium falciparum* multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione," *Journal of Biological Chemistry*, vol. 284, no. 12, pp. 7687–7696, 2009.
38. Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA. Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Mol Microbiol.* 2005 Apr; 56(2):323-33. Review. PubMed PMID: 15813727.
39. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, *et al.* (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science* 325: 1680–1682.
40. H. Zhang, E. M. Howard, and P. D. Roepe, "Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast," *Journal of Biological Chemistry*, vol. 277, no. 51, pp. 49767–49775, 2002.
41. A.B. Singh Sidhu, D. Verdier-Pinard, and D.A. Fidock, "Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations," *Science*, vol. 298, no. 5591, pp. 210–213, 2002.
42. Slater AF. Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*. *Pharmacol Ther.* 1993 Feb-Mar;57(2-3):203-35. Review. PubMed PMID:8361993.
43. Klonis N, Tan O, Jackson K, Goldberg D, Klemba M, Tilley L. Evaluation of pH during cytostomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*. *Biochem J.* 2007 Nov 1; 407(3): 343-54. PubMed PMID: 17696875; PubMed Central PMCID: PMC2275073.
44. Reeves DC, Liebelt DA, Lakshmanan V, Roepe PD, Fidock DA, Akabas MH. Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms. *Mol Biochem Parasitol*. 2006 Dec;150(2):288-99. PubMed PMID: 17014918; PubMed Central PMCID: PMC1687154.
45. Sugioka Y, Suzuki M. The chemical basis for the ferriprotoporphyrin IX-chloroquine complex induced lipid peroxidation. *Biochim Biophys Acta.* 1991 May 24;1074(1):19-24. PubMed PMID: 2043670.
46. Philip J. Rosenthal, Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery, Springer Science & Business Media, 01-Apr-2001, Pg 97.
47. Coronado LM, Nadovich CT, Spadafora C. Malarial hemozoin: from target to tool. *Biochim Biophys Acta.* 2014 Jun;1840(6):2032-41. 10.1016/j.bbagen.2014.02.009. Review. PubMed PMID: 24556123; PubMed Central PMCID: PMC4049529.
48. Li J, Chen J, Xie D, Monte-Nguba SM, Eyi JU, Matesa RA, Obono MM, Ehapo CS, Yang L, Lu D, Yang H, Yang HT, Lin M. High prevalence of pfmdr1 N86Y and Y184F mutations in *Plasmodium falciparum* isolates from Bioko Island, Equatorial Guinea. *Pathog Glob Health.* 2014 Oct;108(7):339-43. doi: 10.1179/2047773214Y.0000000158. PubMed PMID: 25348116; PubMed Central PMCID: PMC4241786.
49. Purfield A, Nelson A, Laoboonchai A, Congpuong K, McDaniel P, Miller RS, Welch K, Wongsrichanalai C, Meshnick SR. A new method for detection of pfmdr1 mutations in *Plasmodium falciparum* DNA using real-time PCR. *Malar J.* 2004 May 7;3:9. PubMed PMID: 15132750; PubMed Central PMCID: PMC420476.
50. Nkhoma S, Nair S, Mukaka M, Molyneux ME, Ward SA, Anderson TJ. Parasites bearing a single copy of the multi-drug resistance gene (pfmdr-1) with wild-type SNPs predominate amongst *Plasmodium falciparum* isolates from Malawi. *Acta Trop.* 2009 Jul;111(1):78-81. doi: 10.1016/j.actatropica.2009.01.011. PubMed PMID: 19426667; PubMed Central PMCID: PMC2746100.
51. Ursing J, Zakeri S, Gil JP, Björkman A. Quinoline resistance associated polymorphisms in the pfcrt, pfmdr1 and pfmrp genes of *Plasmodium falciparum* in Iran. *Acta Trop.* 2006 Mar;97(3):352-6. PubMed PMID: 16490179.
52. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Surin J. The detection of pfcrt and pfmdr1 point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. *Malar J.* 2012 Aug 1;11:251. doi: 10.1186/1475-2875-11-251. PubMed PMID: 22853645; PubMed Central PMCID: PMC3493286].
53. Pulcini S, Staines HM, Lee AH, Shafik SH, Bouyer G, Moore CM, Daley DA, Hoke MJ, Altenhofen LM, Painter HJ, Mu J, Ferguson DJ, Llinás M, Martin RE, Fidock DA, Cooper

RA, Krishna S. Mutations in the *Plasmodium falciparum* chloroquine resistance transporter, PfCRT, enlarge the parasite's food vacuole and alter drug sensitivities. *Sci Rep*. 2015 Sep 30;5:14552. doi: 10.1038/srep14552. PubMed PMID: 26420308; PubMed Central PMCID: PMC4588581.

54. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Surin J. The detection of pfcrt and pfmdr1 point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. *Malar J.* 2012 Aug 1;11:251. doi: 10.1186/1475-2875-11-251. PubMed PMID: 22853645; PubMed Central PMCID: PMC3493286.
55. Tu Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nat Med.* 2011 Oct 11;17(10):1217-20. doi: 10.1038/nm.2471. PubMed PMID: 21989013.
56. Miller LH, Su X. Artemisinin: discovery from the Chinese herbal garden. *Cell.* 2011 Sep 16;146(6):855-8. doi: 10.1016/j.cell.2011.08.024. PubMed PMID: 21907397; PubMed Central PMCID: PMC3414217.
57. Meshnick SR. Artemisinin: mechanisms of action, resistance and toxicity. *Int JParasitol.* 2002 Dec 4;32(13):1655-60. Review. PubMed PMID: 12435450.
58. Cui L, Su XZ. Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev Anti Infect *Ther*. 2009 Oct;7(8):999-1013. Doi: 10.1586/eri.09.68. Review. PubMed PMID: 19803708; PubMed Central PMCID: PMC2778258.
59. Kansanen E, Jyrkkänen HK, Levonen AL. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic Biol Med.* 2012 Mar 15;52(6):973-82. doi: 10.1016/j.freeradbiomed.2011.11.038. Review. PubMed PMID:22198184.
60. Zhang F, Gosser DK Jr, Meshnick SR. Hemin-catalyzed decomposition of artemisinin (qinghaosu). *Biochem Pharmacol* 1992;43:1805–1809. [PubMed: 1575774]
61. Krishna S, Uhlemann AC, Haynes RK. Artemisinins: mechanisms of action and potential for resistance. *Drug Resist Updat* 2004;7:233–244. [PubMed: 15533761]
62. Haynes RK, Krishna S. Artemisinins: activities and actions. *Microbes Infect* 2004;6:1339–1346. [PubMed: 15555542]
63. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci U S A.* 1994 Oct 11;91(21):9926-30. PubMed PMID: 7937919; PubMed Central PMCID: PMC44930.
64. Ma Q. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol.* 2013;53:401-26. doi: 10.1146/annurev-pharmtox-011112-140320. Review. PubMed PMID: 23294312; PubMed Central PMCID: PMC4680839.
65. Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol.* 2013 Jan 18;1:45-9. doi: 10.1016/j.redox.2012.10.001. Review. PubMed PMID: 24024136; PubMed Central PMCID: PMC3757665.
66. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes Cells.* 2011 Feb;16(2):123-40. doi: 10.1111/j.1365-2443.2010.01473.x. Review. PubMed PMID: 21251164.
67. Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, Baban D, Nye E, Stamp GW, Wolhuter K, Stevens M, Fischer R, Carmeliet P, Maxwell PH, Pugh CW, Frizzell N, Soga T, Kessler BM, El-Bahrawy M, Ratcliffe PJ, Pollard PJ. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell.* 2011 Oct 18;20(4):524-37. doi:0.1016/j.ccr.2011.09.006. PubMed PMID: 22014577; PubMed Central PMCID: PMC3202623.
68. Yueh MF, Tukey RH. Nrf2-Keap1 signaling pathway regulates human UGT1A1 expression in vitro and in transgenic UGT1 mice. *J Biol Chem.* 2007 Mar 23;282(12):8749-58. PubMed PMID: 17259171.
69. Mackenzie PI, Hu DG, Gardner-Stephen DA. The regulation of UDP-glucuronosyltransferase genes by tissue-specific and ligand-activated transcription factors. *Drug Metab Rev.* 2010 Feb;42(1):99-109. Doi: 10.3109/03602530903209544. PubMed PMID: 20070244.
70. Bock KW, Köhle C. UDP-glucuronosyltransferase 1A6: structural, functional, and regulatory aspects. *Methods Enzymol.* 2005;400:57-75. Review. PubMed PMID: 16399343.
71. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999 Jan 1;13(1):76-86. PubMed PMID: 9887101; PubMed Central PMCID: PMC316370.
72. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol.* 2004 Aug;24(16):7130-9. PubMed PMID: 15282312; PubMed Central PMCID: PMC479737.
73. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes Cells.* 2011 Feb;16(2):123-40. doi: 10.1111/j.1365-2443.2010.01473.x. Review. PubMed PMID: 21251164.
74. WHO/HTM/GMP/2016.11
75. Visser BJ, Wieten RW, Kroon D, Nagel IM, Bélard S, van Vugt M, Grobusch MP. Efficacy and safety of artemisinin combination therapy (ACT) for non-falciparum malaria: a systematic review. *Malar J.* 2014 Nov 26;13:463. doi:

10.1186/1475-2875-13-463. Review. PubMed PMID: 25428624; PubMed Central PMCID: PMC4258384

76. Rogers WO, Sem R, Tero T, Chim P, Lim P, Muth S, Socheat D, Ariey F, Wongsrichanalai C. Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. *Malar J*. 2009 Jan 12;8:10. doi: 10.1186/1475-2875-8-10. PubMed PMID: 19138388; PubMed Central PMCID: PMC2628668

77. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. *Cell*. 2006 Feb 24;124(4):755-66. Review. PubMed PMID: 16497586.

78. Khalil HS, Goltsov A, Langdon SP, Harrison DJ, Bown J, Deeni Y. Quantitative analysis of NRF2 pathway reveals key elements of the regulatory circuits underlying antioxidant response and proliferation of ovarian cancer cells. *J Biotechnol*. 2015 May 20;202:12-30. doi: 10.1016/j.jbiotec.2014.09.027. PubMed PMID: 25449014