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Review Article

ALTERATION IN *PLASMODIUM FALCIPARUM* PROTEOME UPON TREATMENT WITH VARIOUS ANTI-MALARIAL DRUGS

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Abstract: *Plasmodium falciparum*, deadliest among all malaria parasites, is creating new challenges by developing resistance against most of the currently used drugs. Chemotherapy is the most promising way to combat malaria due to the absence of any potential vaccine candidate. In the current scenario it becomes necessary to understand the mechanism of action of these anti-malarials and the associated mechanism of resistance.

Proteomic technologies open up a novel direction to unravel the biology of malaria parasite as well as specific changes associated during treatment with various anti-malarials. Various advanced proteomic tools are available which gives elaborative information about the particular response associated with any drug exposure, which would help in the identification of affected pathway. By using this information, new anti-malarial drug or a combination of two anti-malarials having different mode of action may be employed for the treatment of drug resistant malaria. This review mainly highlights the application of proteomic technologies in deciphering the mode of drug action on the malaria parasite *Plasmodium falciparum* while summarizing the use of proteomic technologies in other aspects of malaria.

Key Words: *Plasmodium falciparum;* Drug resistance; Anti-malarial drugs; Biomarkers; Differential proteomics.

1. Introduction

Malaria is a major health burden in tropical and sub tropical regions of the world. Despite immense efforts for the treatment and campaign regarding prevention and control of this parasitic disease, it is still a rampant problem worldwide. Life cycle of *Plasmodium* is complex which involves vertebrate as well as invertebrate host systems to complete its biological cycle. Sexual cycle of development occurs in female *Anopheles* mosquito while asexual cycle takes place inside human host. The overall life cycle of *Plasmodium* involves diverse stages among which drastic morphological changes has been observed (Bautista *et al.*, 2014). Amongst all species, the most severe form of malaria is caused by *Plasmodium falciparum* which affects multiple organs of human host (Siqueira-Batista *et al.*, 2012). The most significant challenge associated with malaria chemotherapy is ever increasing drug resistance in *Plasmodium falciparum* (Cooper and Carucci, 2004). Simultaneously problems linked with the development of any promising vaccine is another hindrance for the prevention of malaria (Playfair *et al.*, 1990). Artemisinin and its derivatives are the most promising anti malarial drugs known these days especially after the development of chloroquine resistance among parasites. But in the southeast regions of Asia resistance in *Plasmodium falciparum* towards this vital class of drug has also emerged (Ariey *et al.*, 2014). In the current scenario it has been suggested

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by WHO to use artemisinin based combination therapies instead of monotherapy.

Proteomics is an important tool to understand the parasite biology, its interaction with host system and mechanism of drug action (Prieto *et al.*, 2008). Additionally, by using proteomic technologies we can explore new drug and vaccine targets in parasite as well as unravel the mechanism of drug resistance. It also gives information about the specific biology of each developmental stage of the parasite (Cooper and Carucci, 2004). Moreover, quantitative as well as qualitative changes in protein expression pattern of parasite (under some particular condition) can also be identified by applying advanced proteomic technologies (Nirmalan *et al.*, 2004).

The term "proteomics" was first described in 1995 (Anderson and Anderson, 1996) and was defined as the large scale analysis of proteome expressed by a particular cell or an organism which would significantly help in understanding the gene function (Pandey and Mann, 2000). The aim of proteomics cover both the identification and localization of proteins in a cell by using a complete three dimensional map through various technologies (Graves and Haystead, 2002). As such, the technology holds immense promise in understanding disease biology and the response to the use of drugs. In this article, such applications in malaria biology have been reviewed and scope of future research identified.

2. Potential applications of proteomics in malaria

Proteomics has opened up various dimensions in malaria research. The first proteomic study in malaria was carried out three decades ago for the analysis of differential protein expression in parasite after mefloquine treatment (Pinswasdi *et al.*, 1987). This was done using 2D gel electrophoresis but further identification of proteins was again a challenging issue at that time due to unavailability of whole genome sequence of *P. falciparum*. Later this complexity was solved because of completion of whole genome sequencing of *P. falciparum* (Gardner *et al.*, 2002) coupled with advancement in mass spectrometry techniques for the identification of proteins (Aebersold and Mann, 2003). Since then the scope has widened and the various possibilities are summarized below.

2.1. Stage specific proteome of Plasmodium

The life cycle of *Plasmodium* involves various developmental stages across both the sexual and asexual phases in which significant structural changes have been observed. In the last decade, two groups (Florens *et al.*, 2002; Lasonder *et al.*, 2002) have simultaneously explored the stage specific expression of *Plasmodium falciparum* proteome in order to understand the specific function of each stage (Figure 1) using MudPIT and LC-MS/MS based techniques. Later few other groups have also explored the proteome of parasite in different developmental stages using other techniques which includes 2D gel electrophoretic based separation of proteins followed by identification through MALDI-TOF MS and MALDI-QIT-TOF-MS (Gelhaus *et al.*, 2005; Kasai and Ikegami-Kawai, 2012). An another study done by Lasonder et al. in 2008 was focussed mainly on the proteomes of different mosquito stages of the parasite (Figure 2) by nano LC-MS/MS technique. The details of the above mentioned studies have been reviewed earlier (Sims and Hyde, 2006; Siqueira-Batista *et al.*, 2012). Sex specific biology of *Plasmodium* was also explored by analyzing the male and female gametocyte proteome of *Plasmodium berghei* (Khan *et al.*, 2005) to understand the process of transmission in mosquitoes and further sexual development of the parasite. It was found that only 69 proteins were common among male and female gametocyte proteome of malaria parasite. Most of the male gametocyte specific proteins are responsible for flagellar motility and replication of genome. Moreover male and female gametocytes express unique kinases and phosphatases which suggests gender specific differential behaviour during signal transduction of malaria parasite (Khan *et al.*, 2005). Later another study was also done by Tao *et al.* (2014) for the identification of sex specific protein markers of stage V gametocyte of *Plasmodium* using proteomic and bioinformatic approaches. These protein markers would help in the development of gametocyte specific biomarkers as well as transmission blocking vaccine candidate (Tao *et al.*, 2014).

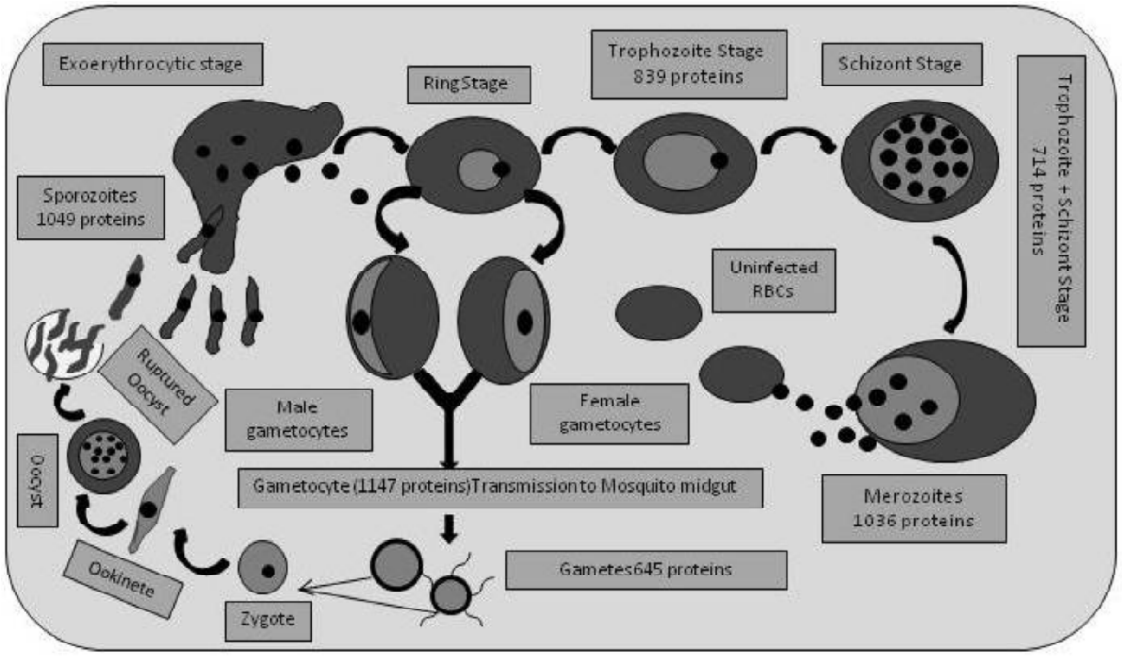

Figure 1: **Diagrammatic representation of the life cycle of malaria parasite depicting various numbers of proteins expressed during different life cycle stages. Proteins expressed during sporozoite, merozoite, trophozoite and gametocyte stages were identified by Florens *et al.* while Lasonder *et al.* has also explored proteins expressed during trophozoite, schizont, gametocyte and gamete stages**

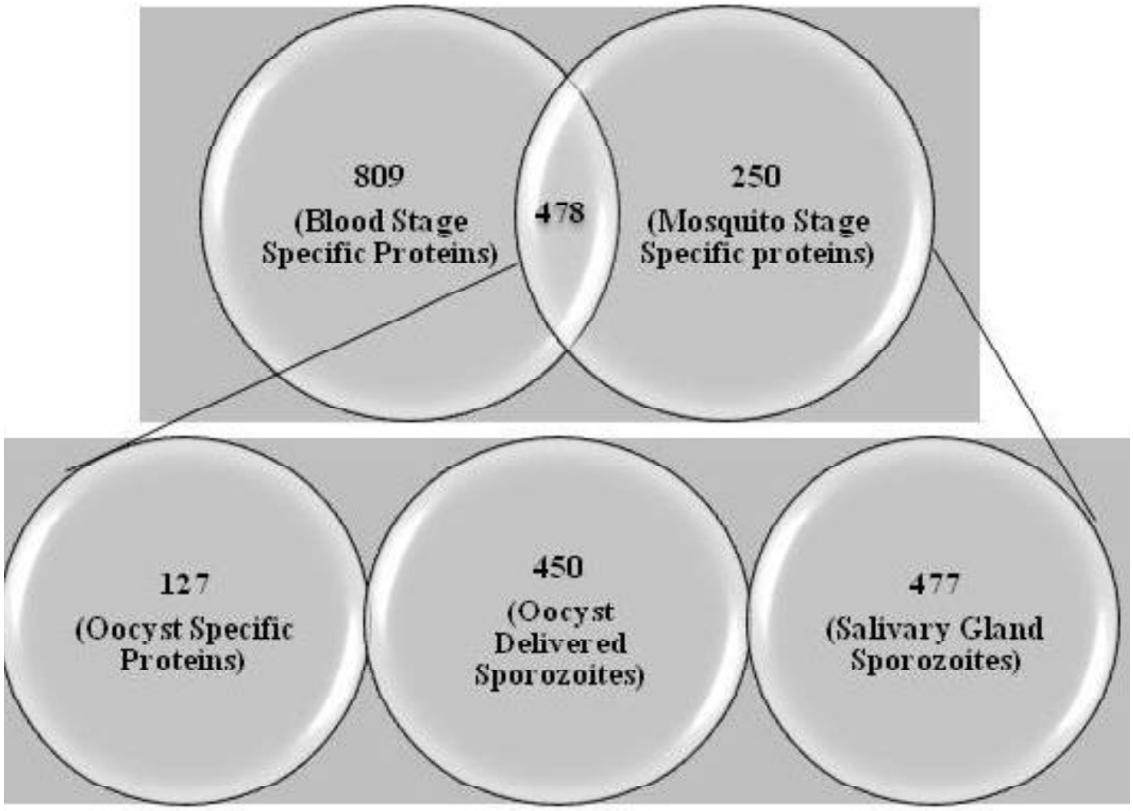

Figure 2: **These Venn diagrams show distribution of 1059 proteins among blood and mosquito stages of *P. falciparum* 3D7. Further distribution of total 728 mosquito stage proteins among oocyst, oocyst derived sporozoite and salivary gland sporozoites are also represented in the Venn diagram**

Apart from *P. falciparum* few other studies have been carried out to explore the proteome of *P. vivax* which would help in the development of novel drug targets and rapid diagnostic tests specific for *P. vivax* (Moreno-Perez *et al.,* 2015; Roobsoong *et al.,* 2011).

2.2. *Organelle specific proteome of parasite*

Earlier proteomic analysis of parasite was dedicated to unravel the entire cell proteome of the parasite in a particular developmental stage. Based on whole cell proteome data we can explore the stage specific parasite protein as well as we can compare the function of different developmental stages of the parasite. But to understand the specific structure and function of a cell organelle we have to identify the proteome at organelle level. In this context, few studies were carried out which covers the nucleus, food vacuole, Maurer's cleft, membranes of infected RBCs, rhoptry and microneme associated proteins (Table 1). As expected, the nucleus revealed a large number of proteins compared to other organelle. The need of the hour is to analyze these proteins and their interactome to identify unique drug targets that can be explored.

Table 1
Total proteins identified in different organelles of parasite

Parasite cell organelle	*No. of Proteins expressed*	*References*
Nucleus	1273	(Oehring *et al.*, 2012)
Food vacuole	116	(Lamarque *et al.,* 2008)
Maurer's cleft	78	(Vincensini *et al.,* 2005)
Membrane of iRBCs	36	(Florens *et al.,* 2004)
Rhoptry	36	(Sam-Yellowe *et al.,* 2004)
Microneme	345	(Lal *et al.,* 2009)

2.3. *Identification of biomarkers*

Proteomic technologies were widely used to decipher biomarker in various diseased conditions like dengue (Albuquerque *et al.,* 2009), leishmaniasis (Rukmangadachar *et al.,* 2011), SARS (Chen *et al.,* 2004) and leptospirosis (Srivastava *et al.,* 2012). Malaria infected patients also show alteration in their serum proteome compared to healthy individuals (Ray *et al.,* 2012). Such change in the serum proteome is very helpful for the identification of specific biological markers which might play important role in studying disease pathogenesis and changes in the immune response during infection. During diseased state sudden change in expression pattern of serum proteins gives direct information about the severity of disease (Ray *et al.,* 2011).

Ray *et al.* in 2012 explored the biomarkers for *falciparum* and *vivax* malaria (Ray *et al.,* 2012). This study involved both conventional 2D gel electrophoresis and DIGE based gel electrophoresis to interpret the differentially expressed proteins in patients suffering with both types of malaria relative to healthy control. In case of *falciparum* malaria, 22 spots were differentially expressed by using conventional 2D gel electrophoresis. Further 12 spots were identified through MALDI-TOF MS analysis; among which 7 proteins were up regulated and 5 were down regulated. However DIGE based gel electrophoresis showed 121 differentially expressed spots. Further analysis through MALDI-TOF MS identified 63 spots while 58 spots remain unidentified. These 63 identified spots corresponded to 30 differentially expressed proteins among which 14 and 16 proteins were up regulated and down regulated respectively. Analysis of the proteins expressed among healthy subjects and both types of malaria patients consistently indicated the differential expression of "haptaglobulin" as well as "serum amyloid A", suggesting that these proteins might well be used as biomarkers. Further studies with larger data set are indeed needed and this aspect offers scope to proteomics researchers to combat malaria.

Comparison of the differentially expressed proteins in *falciparum* and *vivax* malaria has shown that around half of the significantly altered proteins were common however the magnitude of change was different (Ray *et al.,* 2012). Around 5 proteins were specific to each group and not found in other group while 19 common proteins showed differential expression. Amongst these 19 proteins, alpha 2-HS-glycoprotein and serotransferrin precursor (transferrin) showed reverse trend in *falciparum* and *vivax* malaria. In case of *falciparum* malaria, expression of calcium binding protein 39, regulator of G-protein

signalling 7, calpain 10, transthyretin and serum paraxonase were specifically altered whereas expression of vitamin D-binding protein, alpha-2-macroglobulin, ceruloplasmin, fibrinogen beta chain precursor and serum amyloid P were modified in case of *vivax* malaria.

Comparative analysis of *falciparum* and *vivax* malaria also identified 43 differentially expressed spots in case of *falciparum* infection (Ray *et al.*, 2012). Further analysis by MALDI-TOF/TOF MS led to the identification of 13 proteins, of which 5 were up regulated and 8 were down regulated. In *Plasmodium falciparum* infected patients, five up regulated proteins were identified as serum amyloid A, interleukin-17E precursor, alpha-1-antitrypsin, ficolin 3 precursor and C region of Ig kappa chain whereas down regulated proteins were identified as apolipoprotein E, alpha-2-HS glycoprotein, serotransferrin precursor, leucinerich alpha-2-glycoprotein, vitamin D binding protein, alpha-1-antichymotrypsin, heptaglobulin and AMBP protein. Based on these findings biomarker specific to malaria or more specifically to *vivax* and *falciparum* malaria could be developed and needs heightened attention. The traditional determination of malaria by using microscopy of blood smears is a well established technique but it requires highly experienced person as well as in poor endemic areas operating cost for microscopy is high (Mockenhaupt *et al.*, 2000; Payne, 1988) with the added limitation of detection of mixed species infection. Hence, point-of-care tools could be developed using these biomarkers, which would help in the detection of specific type of malaria as well as mixed-species infections; additionally diagnosis of very low level of parasitemia can also be achieved. These areas need immediate attention.

Indeed, these days some protein biomarkers are being used in rapid diagnostic tests (RDTs) for the detection of malaria, which includes *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH), *Plasmodium falciparum* histidine rich protein II (*Pf*HRP2), *Plasmodium* aldolase and *Plasmodium* glutamate dehydrogenase (GDH) (Mathema and Na-Bangchang, 2015). Use of these RDTs in clinic provides advantages in terms of cost, time and sensitivity. However, there are certain limitations too - they cannot analyze frozen samples and are also very sensitive towards moisture and high temperature. Diagnosis is also manual and depends on the experience of performer.

Hence in this scenario, development of highly efficient biosensors are required which can use the above mentioned serum protein markers for the detection of specific type of malaria.

2.4. *Identification of the mechanism of drug resistance*

Drug resistance has proved to be the single most barrier to treat malaria of late, and this aspect of malaria has gained worldwide attention. Proteomic technologies have also been employed to detect mechanisms underlying the drug resistance in *Plasmodium falciparum*. Koncarevic et al. performed comparative proteome analysis of chloroquine sensitive and resistant *Plasmodium falciparum* strains using SELDI-TOF-MS (Koncarevic *et al.*, 2007). Four different *P. falciparum* strains *3D7, HB3, K1* and *Dd2* were selected for this study, among which *3D7* and *HB3* were chloroquine sensitive while *K1* and *Dd2* were chloroquine resistant. After chloroquine treatment on both sensitive and resistant parasite strains, SELDI-TOF protein spectra were obtained; among which ten protein peaks were identified as markers. Amongst these, six protein peaks showed up regulation whereas four showed down regulation. Three proteins were further identified as EXP-1 (circumsporozoite related antigen), haemoglobin β and δ subunit and haemoglobin α subunit. Level of EXP-1 was higher in sensitive strains than resistant; however after chloroquine exposure resistant strains showed up regulation of this protein but sensitive strain showed down regulation of EXP-1.

These unique changes in EXP-1 suggest that it can be an excellent marker protein apart from causing cellular damage after drug exposure. However, further studies (eg- pull down assay, gene silencing) are required to predict the direct correlation of this protein in CQ resistance.

Other proteins namely human haemoglobin α, β (or δ) were found to be more abundant in sensitive than in resistant strains. From this finding it can be concluded that less concentration

of haemoglobin in resistant parasite strain will lead to the lesser concentration of free toxic heme. Hence higher CQ concentration is required to achieve lethal concentration of free heme for parasite killing. Given the importance and necessity of suitable drug to treat malaria; the rest of this article deals with this aspect in details.

3. Unravelling the mechanism of drug action in response to drug exposure

Understanding the mechanism of action of anti malarial drugs is an essential prerequisite to explore the drug resistance in parasite as well as for the development of new drugs which will be used for either monotherapy or combination therapy. Due to drug exposure RNA expression profile of parasite changes leading to corresponding change in the expression of proteins. However, few new modifications at the level of proteome also occur.

This approach has been employed in various diseases to explore the mechanism of drug action. One example of such study involved differential analysis of human HepG2 cells proteome upon exposure of doxorubicin (Hammer *et al.*, 2010) as monitored by 2D-DIGE and SILAC techniques followed by MS based analysis. It was found that doxorubicin down regulates the protein responsible for DNA replication and protein synthesis, while it up regulates the proteins responsible for DNA damage control, oxidative stress management and multiple forms of keratin (a structural protein). Hence, from this study it was concluded that doxorubicin mainly affects proteins involved in DNA damage control, protein synthesis, electron transport function and tumour growth.

Another study was performed on pancreatic ductal carcinoma cells proteome upon combined exposure of gemcitabine and trichostatin A (Cecconi *et al.*, 2005). Gemcitabine interfered with DNA synthesis and trichostatin A interfered with histone acetylation process. These drugs together showed significant co-operativity and suppressed tumour formation while activating apoptosis of the cells. This data was further supported by comparative proteomic studies of control and drug treated cell lines using 2D gel electrophoresis followed by MALDI-TOF and Q-TOF MS analysis. This study confirmed that gemcitabine and trichostatin A mainly affects cell proliferation and apoptotic death of cells.

A separate study involved monitoring of proteomic alterations on embryonic kidney cell line post mycophenolic acid treatment (Qasim *et al.*, 2011) to see its effect. In this study drug treated and control proteins were separated by 2D gel electrophoresis followed by analysis of differentially expressed proteins using QTOF-MS/MS. Result of this experiment suggested that mycophenolic acid mostly affects proteins responsible for formation of cytoskeleton, structure and dynamics of chromatin and energy formation. These diverse studies indicated that proteomics is a viable approach to investigate the action of drugs on malarial proteome as well. Indeed, different proteomic approaches were employed to understand the mode of action for the anti-malarial drugs and Figure 3 displays a timeline of such investigations. The relevant knowledge relating to some milestone drugs are reviewed here.

3.1. Quinoline

Quinolines are the most successful class of anti-malarial drugs; however, their mechanisms of action are not completely understood. The first study was carried out by Graves *et al.* in 2002 to explore the mechanism of action of quinoline drugs using functional proteomics based approach. This class of drugs share few structural similarities to the purine nucleotide. Hence it was proposed that quinolines might interact with purine binding proteins. So, in the study performed by Graves *et al.* (2002) purine binding proteome were isolated by using affinity matrix ã-phosphate linked ATP-Sepharose. It was found that human purine binding proteins ALDH1 (aldehyde dehydrogenase 1) and QR2 (Quinone reductase 2) directly interacts with quinolines. Among these two proteins QR2 plays role during metabolic detoxification of quinines, which is found in all respiring plant and animal cells and constitutes a large class of significantly toxic compounds (Dinkova-Kostova and Talalay, 2000; Long and Jaiswal, 2000). Hence in this study it was concluded that quinolines induces oxidative stress inside RBCs by hampering quinone

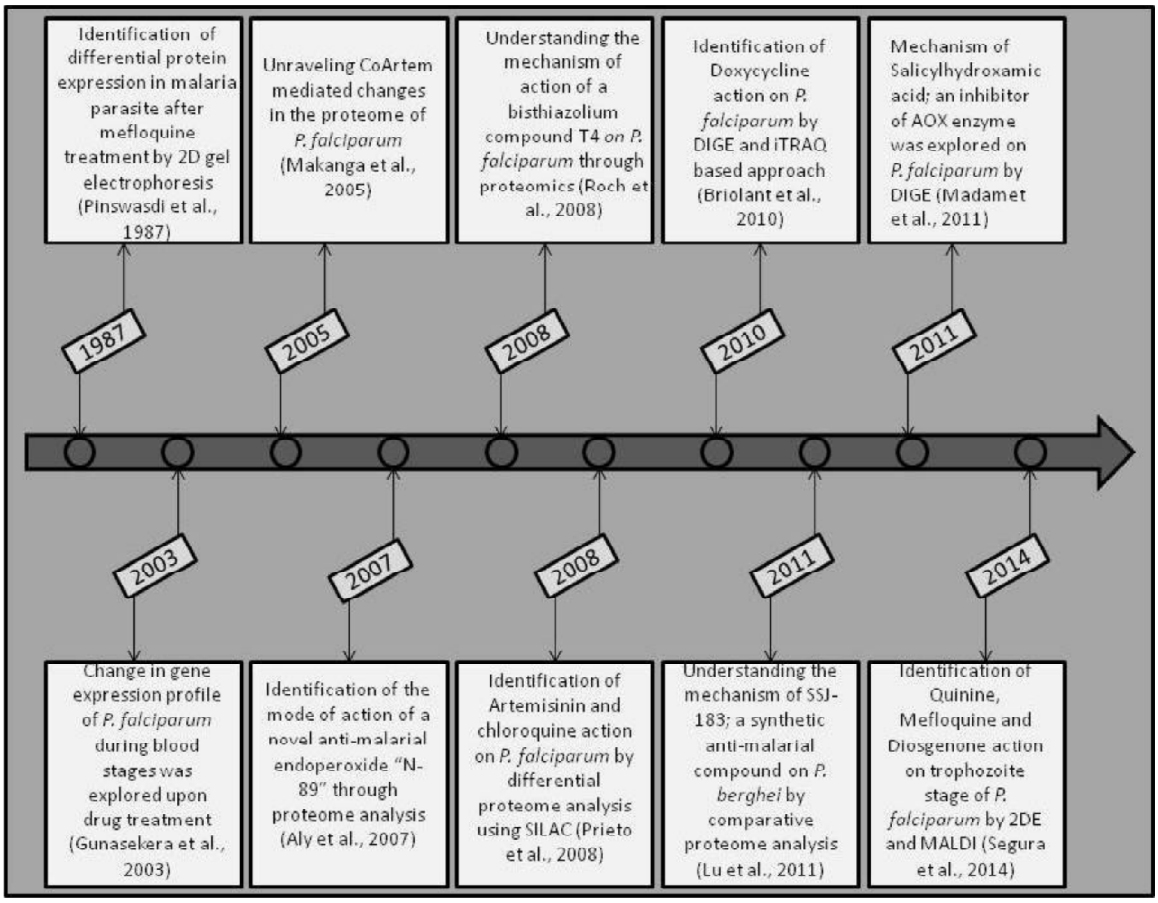

Figure 3: **Timeline to show major milestones in the identification of mechanism of anti-malarial action on *Plasmodium* using different proteomics based approach**

reductase activity and as a result it creates unfavourable environment for the parasite. In the case of malaria, *Plasmodium* generates oxidative stress within RBCs due to haemoglobin digestion and this environment becomes toxic for parasite in the absence of host anti-oxidant enzymes (QR2). In case of another enzyme ALDH1, quinolines play role as a weak inhibitor preventing the oxidation of aldehydes to carboxylic acids, thus producing unfavourable environment for the parasite. However, this enzyme plays role during generation of retinoic acid from retinaldehyde in the eye and upon treatment with chloroquine its accumulation takes place within eye and skin of the patients. This accumulated chloroquine further interacts with ALDH1 and interferes during formation of retinoic acid. Hence treatment with high concentration of chloroquine may cause retinopathy, which is a serious side effect. However, from this study it was evident that by the application of novel proteomic techniques, like proteome mining, all drug targets can be identified. Further compounds with preference for QR2 over ALDH1 can be identified as a good anti-malarial drug.

3.2. Coartem

The anti-malarial action of a novel compound CoArtem, which is chemically formed by the combination of artemether (ARM) and lumefantrine (LUM) was identified through proteomic strategy by Makanga *et al.* (Makanga *et al.*, 2005). Artemisinin affects various cell organelles of the parasite like mitochondria, ribosome and nucleus (Gu *et al.*, 1984, 1986; Maeno *et al.*, 1993) which shows its involvement in various pathways of the parasite. However, lumefantrine is structurally similar to few other anti-malarials and hence may show its anti-malarial activity through interaction with heme present in the food vacuole of parasite (Biagini *et al.*, 2003).

In ARM treated parasites 29 protein spots displayed down regulation with more than 3 fold intensity, among which 2 protein spots showed the same trend followed by LUM treatment. Scrutiny of protein spots followed by ARM and LUM treatment have shown three fold up regulation of 22 and 41 proteins, respectively. In this study it has been observed that artemisinin and lumefantrine show contrasting effect on glycolytic enzymes but both drugs affect stress response proteins in a similar manner. Followed by ARM treatment a number of glycolytic enzymes were three fold down regulated while LUM treatment showed up regulation of these enzymes with the same magnitude. ARM treatment leads to the up regulation of a membrane bound calcium binding protein which proves the alteration of calcium binding protein of the parasite. Similarly LUM treatment also shows up regulation of the same protein. This study also revealed that ARM treatment leads to the down regulation of enzymes responsible for protein synthesis, nucleic acid metabolism, parasite respiration and amino acid biosynthesis, which supports the previous data. Moreover a number of proteins were up regulated on treatment with ARM, like membrane bound calcium binding protein, aspartic proteinase, heat shock proteins (HSP60, HSP70 & HSP90) and two proteins responsible for protein metabolism.

3.3. N-89

A novel anti-malarial N-89, an endoperoxide group of compound has shown good anti-malarial activity and selectivity. Aly *et al.* (Aly *et al.,* 2007) has applied a distinct approach to identify the mechanism of N-89 action through proteome analysis. They developed *in vitro* an N-89 resistant strain of parasite known as NRC-10. Subsequently a comparative proteome analysis after drug exposure was done among N-89 sensitive and resistant strains of the parasite. Post N-89 treatment 12 spots were up regulated and 14 spots were down regulated in NRC-10 strain while one new spot was also observed. Up regulated proteins include MSP-7 precursor, endoplasmic reticulum resident calcium binding protein, DNA J domain protein, fructose-bisphosphate aldolase and heat shock protein while down regulated proteins comprise actin, 14-3-3 protein homologue, phosphoethanolamine N-methyl transferase, enolase, ornithine aminotransferase, S-adenosylmethionine synthetase, Glyceraldehyde 3-phosphate dehydrogenase, chaperonin cpn 60 and uridine phosphorylase. Overall function of these proteins is associated with glycolytic pathway and metabolism of protein and lipid (Aly *et al.,* 2007).

3.4. Chloroquine and artemisinin

An extensive differential proteome analysis was done by Prieto *et al.* in 2008 to understand the molecular mechanism of two important antimalarials - chloroquine and artemisinin (Prieto *et al.,* 2008). SILAC based technique followed by LC-MS/MS was applied for the qualitative as well as quantitative analysis of differential proteome expression. In this study *in vitro* culture of *Plasmodium falciparum* was treated with $2xIC_{50}$ concentration of chloroquine and artemisinin in trophozoite stage for 12 hrs.

In chloroquine treated parasites, total 889 proteins were identified among which 41 proteins were up regulated and 14 were down regulated. Though in artemisinin treated parasite total 804 proteins were identified, in which 38 were up regulated and 8 were down regulated. Additionally a comparative analysis among chloroquine and artemisinin treated parasite was also performed. Overall 16 proteins were specifically up regulated and 6 were specifically down regulated after chloroquine treatment; while in artemisinin treated parasites 18 proteins and 5 proteins were particularly up regulated and down regulated, respectively. A conserved protein PFC0135c of parasite showed contrasting result after treatment with both the drugs. Artemisinin treatment led to the down regulation of PF0135c by 0.28 fold while chloroquine treatment showed up regulation by > 5 fold. Total 750 proteins were identified in both the treated groups. Among which 680 proteins have shown the same trend. However, remaining proteins displayed the unique expression pattern for each treated groups.

3.4.1. Effect of chloroquine

The above study (Prieto *et al.,* 2008) concluded that in chloroquine treated parasites mostly up

regulated proteins were nucleus specific and showed interaction with chromosome. Few proteins responsible for translocation also showed altered expression. However, identification of particular pathway was difficult due to the presence of large number of affected hypothetical proteins.

However, a previous study carried out by Gunasekera et al. on the differential expression of mRNA followed by chloroquine treatment also showed a high level of similarity to the previous study (Gunasekera *et al.,* 2003). Direct correlation; however, cannot be done among transcriptome and proteome profile due to different experimental conditions.

3.4.2. Effect of artemisinin

Artemisinin is the most important drug after the emergence of chloroquine and sulphadoxine/ pyrimethamine resistance in *Plasmodium falciparum* (Kuhn and Wang, 2008). However; like quinine which is another important class of anti-malarial, the exact mechanism of artemisinin action is also not clear (Lelievre *et al.,* 2007; Meshnick, 2002). The peculiar chemical structure of artemisinin is 1,2,4 trioxane structure having endoperoxide bridge, which is an essential feature for its anti-malarial activity. Earlier it has been proposed that the endoperoxide bridge reacts with reduced heme and Fe^{2+} which forms free radical and further reacts with PfATP6. Prieto *et al.* (2008) has shown the down regulation of PfATP6 after artemisinin treatment. However proteins responsible for nucleotide and nucleic acid metabolism, transportation and secretion were up regulated. Moreover, the expression of multidrug resistance gene (pfmdr1) was up regulated in case of both artemisinin and chloroquine treated parasite (Prieto *et al.*, 2008).

3.5. T4 (bisthiazolium compound)

Bisthiazolium compound T4 is a choline analog and exhibits strong potency against drug resistant and sensitive *Plasmodium falciparum* clones. Parasite showed significant morphological changes and cell cycle arrest on exposure of this drug for 30 hrs (Le Roch *et al.,* 2008a). T4 inhibits progress of the parasite from trophozoite to schizont stage. Using proteomics based approach to identify the mechanism of T4 action, a total of 889 proteins were identified, among which drug treated and untreated parasites showed 560 and 571 proteins respectively (Le Roch *et al.,* 2008a). Most of the up regulated proteins in T4 treated parasite exhibited their association with food vacuole. According to proteomics data a considerable down regulation of the choline/ ethanolamine-phosphotransferase (PfCPET) was also identified. PfCPET protein is responsible for the last step of phosphatidylcholine synthesis (Le Roch *et al.*, 2008b). In the same study, transcriptome analysis was also performed which in contrast showed no change in the expression of PfCPET at mRNA level. This result suggested the role of post transcriptional modification of the PfCPET protein followed by exposure of T4. Earlier studies have also supported post transcriptional changes in the parasite due to noticeable difference in mRNA and protein expression levels (Hall *et al.*, 2005; Le Roch *et al.,* 2004; Shock *et al.*, 2007).

According to metabolic data, T4 exposure hinders the biosynthesis of phosphatidylcholine while phosphatidylethanolamine or DNA was not affected. This study has also suggested that post T4 exposure, levels of intracellular choline, phosphocholine and CDP-choline decreases. Since phosphatidylcholine is the main component of the malaria parasite membrane and its requirement increases significantly during the developmental cycle of the parasite inside RBCs (Holz, 1977; Vial *et al.*, 2003), the enzyme responsible for its synthesis is an ultimate target for the identification of new anti-malarials. Based on proteomic study by Roch *et al.,* T4 may be a promising anti-malarial candidate in future.

3.6. Doxycycline

Previous studies showed that doxycycline (DOX) is a known inhibitor of protein synthesis mechanism in bacteria (Chopra and Roberts, 2001). In *Plasmodium falciparum*, nucleotide and deoxynucleotide synthesis was hampered by DOX treatment (Yeo *et al.,* 1998). Tetracycline which is another member of the cycline group inhibits mitochondrial protein synthesis (Blum *et al.,* 1984) and pyrimidine synthesis by de novo pathway (Prapunwattana *et al.,* 1988). Similarly

another cycline compound is minocycline, which showed inhibitory effect during transcription of mitochondrial and plastid gene (Lin *et al.,* 2002). Few studies have also reported particular role of cyclines on the *P. falciparum* apicoplast (Dahl *et al.,* 2006; Goodman *et al.,* 2007), though the exact mechanism implicated in plastid regulation at molecular level is not specifically explored. To unravel the mechanism of doxycycline action; Briolant *et al.* (2010) employed DIGE and iTRAQ based techniques. In this study total 64 differentially expressed proteins were explored using both proteomic approaches. Different cell organelles showed distinct proportion of the altered level of proteins; 21% were cytoplasmic, 19% were apicoplast specific, 13% were nucleus and membrane specific while, mitochondria contained 5% of the total and remaining 29% were unknown. Most of the up regulated proteins were cytoplasm, apicoplast and membrane specific while most down regulated proteins were of nuclear and mitochondrial origin. Few metabolic pathways were also affected by DOX exposure. Like tetracycline, direct inhibition of mitochondrial protein synthesis was also observed on treatment with doxycycline. This study shows correlation of DOX exposure on parasite protein synthesis and indicates that mitochondria and apicoplast are most affected organalles by DOX (Briolant *et al.,* 2010).

3.7. SSJ-183

SSJ-183, a synthetic compound which is a derivative of benzo(a)phenoxazine, showed very good anti malarial activity *in vitro* as well as *in vivo* (Schleiferböck *et al.*, 2013). Though being a promising anti-malarial candidate its mechanism of action is not clear. Lu *et al.* (2011) has identified the mechanism of SSJ-183 action, but in contrast to previous proteomic studies they selected *in vivo* rodent malaria model for the analysis. Comparative proteomics data of *P. berghei ANK*A displayed up regulation of two protein spots and down regulation of seven protein spots on treatment with SSJ-183. In down regulated groups three main proteins were identified as heat shock protein, disulphide isomerase precursor and berghepain-2. Down regulation of these proteins gives idea about the mode of SSJ-183 action. Heat shock proteins act as molecular chaperone and perform a critical role in the life cycle of parasite during shift form cold blooded mosquito vector to warm blooded human vector. Disulphide isomerase precursor comes under protein disulphide isomerase family and plays role during reshuffle of both inter as well as intra chain disulphide bonds. Berghepain-2 is a cysteine protease known as falcipains in malaria parasite which plays role during degradation of haemoglobin inside food vacuole of the parasite. Based on these findings all the three down regulated proteins were very important for the survival of parasite and are good targets for anti-malarial drugs (Lu *et al.,* 2011).

3.8. Salicylhydroxamic acid

Salicylhydroxamic acid (SHAM) is a specific inhibitor of alternative oxidase (AOX) enzyme which is found in the mitochondrial electron transport chain of plant, some fungi and protists. *Plasmodium* possesses branched respiratory chain which requires both classical as well as branched respiratory chain in which the later requires an alternative oxidase enzyme. However, this enzyme is not explored in *P. falciparum* till now but SHAM has shown good anti-malarial activity *in vitro*. Moreover, it also exhibits good synergistic effect with atovaquone (a mitochondrial drug) (Murphy and Lang-Unnasch, 1999; Schonbaum et al., 1971) and hyperoxia against the growth of *P. falciparum*. It has shown an IC_{50} value of 246+/-4μM and exposure to the same concentration of drug (IC_{50}) on *P. falciparum* 3D7 for 24-32 hrs leads to the inhibition of parasite growth. 250μM of SHAM alone reduces the parasite growth by 54%; however, it hinders 100% growth of the parasite along with hyperoxia in 48 hrs. To understand SHAM action in malaria parasite one group has done the proteome analysis to identify the alteration in metabolic pathways after SHAM exposure (Almeras *et al.,* 2011). By the use of 2D DIGE technique 18 differentially expressed protein spots were identified between SHAM treated and untreated group. Among these seven protein spots were up regulated while eleven were down regulated. These differentially expressed spots were further identified by LC-MS/MS using NCBInr database against *H. sapiens* and *P. falciparum*. These altered proteins show its role in glycolysis and redox metabolism. The

glycolytic enzymes involve gyceraldehyde-3-phosphate dehydrogensae, L-lactate dehydrogenase, phosphoglycerate mutase and enolase which confer the dependence of parasite on glycolysis for the main source of energy. *Plasmodium* is mainly dependent on anaerobic glycolysis for the production of energy (Van Dooren *et al.,* 2006) and presence of hyperoxic condition is lethal for the parasite. Hence SHAM along with hyperoxia inhibits the growth of *Plasmodium* (Almeras *et al.,* 2011).

3.9. Quinine, mefloquine & diosgenone

In one study, Segura *et al*. (2014) has explored the effect of quinine, mefloquine and diosgenone on trophozoite stage of malaria parasite *Plasmodium falciparum ITG2* strain (Segura *et al.,* 2014). Following changes in parasite proteome has observed in this study after exposure of individual drug:

3.9.1. Quinine

On treatment with quinine malaria parasite showed down-regulation of HSP-70. Previous studies suggested that HSP-70 plays role during development and pathogenesis of malaria (Shonhai *et al.,* 2005). This protein also plays role as a molecular chaperone which assist in protein folding. However, its level of expression differs with type of drug exposure. In case of artemether-lumefantrine it shows up regulation (Makanga *et al.,* 2005) while oxidation of HSP-70 has been observed post chloroquine treatment (Radfar *et al.,* 2008).

Enolase, a glycolytic enzyme showed up regulation under quinine treatment. Similar result was also observed earlier in case of lumefantrine which is structurally relevant to quinine. Nonetheless it showed contrasting behaviour under artemisinin treatment (Makanga *et al.,* 2005). Moreover, quinine also up regulates the expression of parasite calcium binding protein. This protein resides in endoplasmic reticulum of the parasite and might play role during protein trafficking in parasite (La Greca *et al.,* 1997). This protein expressed similar trend under treatment with other anti-malarials too (Aly *et al.,* 2007; Makanga *et al.,* 2005). This over expression of calcium binding protein designates the interference in calcium homeostasis of *P. falciparum* post quinine treatment.

Merozoite surface protein-7, which is a cytosolic protein showed down regulation after quinine exposure. MSP-7 might play role in merozoite development or during release of parasite from RBCs. Translocation of MSP-7 to the surface of merozoite was also reported through interaction with MSP-1, indicating its role during invasion (Pachebat *et al.,* 2001). Down regulation of MSP-7 suggests hindrance in the maturation of parasite, release of parasite from RBCs, merozoite binding and invasion.

3.9.2. Mefloquine

Mefloquine treatment down regulates the expression of eukaryotic translation initiation factor 5a (EIF 5a) while it up regulates the expression of circumsporozoite related antigen EXP-1, merozoite capping protein 1, plasmepsin III, chaperonin and a conserved endoplasmic reticulum resident protein. EIF 5a is a cytoplasmic protein, which is highly conserved in *P. falciparum* and contains a specific amino acid hypusine (Molitor *et al.,* 2004). It has been suggested that hypusine along with deoxyhypusine hydroxylase might be a potential drug target (Kerscher *et al.,* 2010). However in prior studies, this trophozoite stage specific protein has not shown the change in expression on treatment other anti-malarials (Makanga *et al.,* 2005). Circumsporozoite related antigen EXP-1 is a negatively charged protein expressed in endoplasmic reticulum during the asexual phase of the parasites (Ansorge *et al.,* 1997; Spielmann *et al.,* 2006). In contrast to other proteins; behaviour of EXP-1 differs among chloroquine sensitive and resistant strain of *P. falciparum*; it shows up regulation in resistant and down regulation in sensitive strains (Koncarevic *et al.,* 2007). In the study by Segura *et al.* (2014), similar results were found which correlates EXP-1 expression level with mefloquine resistance. Based on these findings EXP-1 can be used to monitor chloroquine and mefloquine resistance. Mefloquine treatment also affects the haemoglobin degradation of parasite by a significant increase in the expression of a food vacuole protein plasmepsin III (Banerjee *et al.,* 2002). Similarly, doxycycline exposure also alters

the level of expression of this protein (Briolant *et al.,* 2010). Chaperonin is another affected protein of parasite under mefloquine exposure. Being a mitochondrial protein it plays role in protein folding inside matrix of mitochondria (Sato and Wilson, 2005). Earlier studies have also suggested altered expression of this protein under treatment with other anti-malarials (Aly *et al.*, 2007; Briolant *et al.,* 2010). An endoplasmic reticulum associated conserved protein of *Plasmodium* was also upregulated in response to mefloquine exposure.

3.9.3. Diosgenone

Diosgenone is chemically a triterpene which inhibits growth during asexual stage of parasite inside RBCs (Lopez *et al.,* 2010). Exposure of this drug in trophozoite stage parasite led to the up regulation of merozoite capping protein 1 (MCP-1), endoplasmic reticulum resident calcium binding protein and merozoite surface protein 7 (MSP 7). Among these, mefloquine treatment has also affected MCP-1 protein which is localized inside nucleus and shows role during invasion. Two other proteins were also affected by treatment with quinine - calcium binding protein has shown up regulation while MSP7 has shown down regulation. These findings have suggested that, treatment with quinine, mefloquine and diosgenone share few similarities in the differential expression of parasite proteome during trophozoite stage.

4. Conclusion

The potential applications of proteomics in malaria research have been outlined here to inspire researchers and draw their attention in combating this deadly disease. It is evident that detailed investigations are required in the areas of stage specific and organelle specific proteomics to identify new drug targets and for further insight into mechanism of drug resistance. Proteomics investigation has thrown up lot of proteins which were differentially regulated in organelle or at different stages, but they need to be validated and analyzed and their interactome investigated. It is also evident that the several candidate biomarkers suggested by proteomics research need validation before they can be taken to a clinical setting. Two of them, haptoglobulin and serum amyloid A, seems to be of great promise and appear consistently in investigations.

However, the major focus of the article has been malarial drugs, their mode of action and drug resistance, which of late has hogged the limelight. Proteomics research has identified several mechanism of drug action, which include (1) targeting purine binding proteins to induce oxidative stress within RBCs creating unfavorable ambience for the parasite (2) targeting heme present in food vacuole of parasite (3) targeting multiple organelle at the same time (4) binding stress response proteins (5) binding and altering membrane based calcium binding protein of parasite (6) targeting enzymes responsible for protein synthesis, nucleic acid metabolism, parasite respiration and amino acid biosynthesis (7) alteration of several heat shock proteins (8) interference with glycolytic pathway and metabolism of protein and lipid (9) up-regulation of proteins involved in transportation and secretion (10) alteration of fatty acid synthesis and membrane re-organization and so on and so forth. In addition, proteomics studies have also shown that expression levels of proteins like circumsporozoite related antigen EXP-1 can be used to monitor emergence of resistance. Such markers must be further investigated and developed.

Quinolines, for example, are effective anti-malarial drugs, but cause side effects like retinopathy. It is evident that proteomics investigation has shown that these drugs might be free from side-effects if they can be modified to make them more specific against some targets like quinone reductase 2 while reducing their ability to bind aldehyde dehydrogenase 1. This provides scope for structural biologists to design such specific drugs while chemists to synthesize them in large yields and low costs. In fact, in cases of many drugs multiple targets were implicated and further investigation in this regard are required to especially identify which targets can lead to drug resistance or toxicity. There is thus a need to delineate such off-targets or undesirable targets and "re-design" existing effective drugs to get rid of resistance by making analogues or modifying them chemically.

Chloroquine was observed to mostly affect nuclear proteins and thus their interaction with chromosome, which however, are yet to be minutely detailed. As a survival strategy, such interrupted interactions might have allowed the parasite to acquire resistance. However, the particular nuclear proteins and their interactions and pathways must be identified to get better insight into drug resistance. The fact that chloroquine was an extremely effective drug before the emergence of resistance indicates that drugs against nuclear proteins might be effective but better avoided to circumvent resistance. This tells us that new drugs should always be subjected to proteomics investigation to identify their targets to again idea as to whether they might bring in resistance at a later stage. In contrast to chloroquine, bisthiazolium compounds have been shown to affect the biosynthesis of phosphatidylcholine while phosphatidylethanolamine or DNA was not affected, with the former affecting membrane composition and are potential anti-malarials.

One disadvantage observed with proteomics investigation was contrasting results for a target between groups investigating a particular case. Such results need to be verified and validated. In some cases two drugs have been shown to result in contrasting regulation for the same target, for example as observed for PF0135c against chloroquine and atemisinin. An interesting question to ponder is what happens when a cocktail of these two drugs are used? Would the target then maintain the same level of expression as in absence of these drugs or would one drug move the expression in one direction, based on its better efficacy? If they neutralize each other out, then the cocktail of such drugs may not be as effective as expected. This is an aspect worth investigating as well.

In summary, proteomics research for malaria holds immense promise and must be pursued with great vigor.

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Abbreviations

1D, one dimensional gel electrophoresis; 2D, two dimensional gel electrophoresis; DIGE, Difference gel electrophoresis; MALDI-TOF MS, Matrix assisted laser desorption/ionization-Time of flight-Mass spectrometry; LC-MS, Liquid chromatography-Mass spectrometry; iTRAQ, Isobaric tag for relative and absolute quantitation.

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