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Use of Radioactive Precursors for Biochemical Characterization the Biosynthesis of Isoprenoids in Intraerythrocytic Stages of *Plasmodium falciparum*

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1. Introduction

Malaria continues to be one of the major threats to human health, affecting 300-500 million people and causing the death of approximately 1 million individuals per year, mostly children under 5 years of age (WHO 2010b). Human malaria is caused by five species of the genus *Plasmodium*, namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*, whereas the latter is found exclusively in the Southeast Asian region (Cox-Singh & Singh 2008). Current estimates suggest that approximately 2.4 billion people are at risk of stable or unstable *Plasmodium falciparum* transmission, similar global estimates are also available for *P. vivax*, and while there is considerably less mortality attributed to this species, its geographical reach is far greater. An estimated 2.9 billion people are at risk for vivax malaria, with an estimated 80 million to 300 million clinical cases annually (Guerra *et al.*, 2010). These global estimates are a direct result of an increasing ability to collate and assimilate large data sets that also allow the monitoring of trends in malaria incidence and parasite prevalence. *P. falciparum* is strongly associated with a potentially fatal form of the disease, although recent reports indicate an underestimation of the severity of *P. vivax* infections (Alexandre *et al.*, 2010). Efforts were made to eradicate malaria and although these were successful over large geographical areas, they did not succeed in tropical Africa or in many parts of Asia. In the past few years, malaria has once again attracted more attention partly because of increasing recognition that the malaria prevalence in sub-Saharan Africa has increased during the past decade. The main cause of the worsened malaria situation recorded in recent years has been the spread of drug-resistant parasites, which has led to rising malaria-associated mortality, especially in east Africa.

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The emergence of resistance occurs due to widespread and indiscriminate use of antimalarials. This fact exerts a strong selective pressure on malaria parasites to develop high levels of resistance. On the other hand, the spread of resistance is due to the existence of a sexual cycle in the invertebrate host where there is genetic exchange.

Antimalarial drug resistance is not the same as malaria treatment failure, which is the absence of success in clearing malarial parasitaemia and/or resolve clinical symptoms even with the administration of an antimalarial. While drug resistance may lead to treatment failure, not all treatment failures are caused by drug resistance. Treatment failure can also be the result of incorrect dosing, problems of treatment adherence, poor drug quality, interactions with other drugs, compromised drug absorption or misdiagnosis of the patient. Apart from leading to inappropriate case management, all these factors may also accelerate the spread of true drug resistance by exposure of the parasites to inadequate drug levels (WHO 2010b).

To assess if a strain is resistant to an antimalarial, the World Health Organization (WHO) recommended some methods: *in vivo* assessment of therapeutic efficacy; molecular genotyping to distinguish between re-infections and recrudescence; *in vitro* studies of parasite susceptibility to drugs in culture and identification of molecular markers.

Among the major antimalarial compounds recommended by WHO for treatment of malaria are the aminoquinolines (chloroquine, amodiaquine, primaquine, quinine, mefloquine), the antifolates (sulfadoxine), diaminopyrimidine (pyrimethamine), sesquiterpene lactones (artemisinin, artemether, artesunate) and some antibiotics (WHO 2010a). In counterpart, with the exception of artemisinin derivatives, there is a widespread drug resistance confirmed to all these drugs in many malaria-endemic regions as shown in figure 1 (Ekland & Fidock 2008).

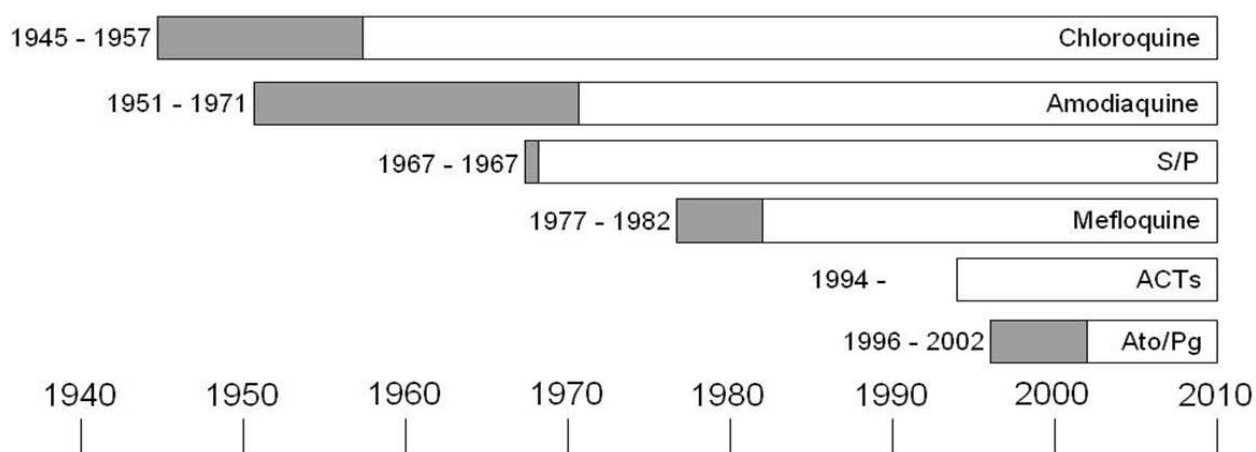


Fig. 1. Emergence of resistance to the principal antimalarials. Each bar represents an antimalarial monotherapy or combination. Years to the left of each bar represent the date the drug was introduced and the first reported instance of resistance. Chloroquine and sulfadoxine/pyrimethamine remained effective for considerable periods after the first reported instances of resistance. Artemisinin-based combination therapies (ACTs); atovaquone/proguanil (Ato/Pg); sulfadoxine/pyrimethamine (S/P), (Adapted from Ekland & Fidock., 2008).

Field trials of artemisinin (Qinghaosu) and its derivatives were first implemented in China in the early 1970s. Artemisinin has a low radical cure rate when used alone in a short course, presumably due to its very short half-life in vivo. Since 1994, artemisinin and its derivatives have been used in combination therapies (ACTs). More recently, in reports about the molecular marker SERCA-type PfATPase6 associated to artemisinin-derivate resistance was described as possible target of the drug (Eckstein-Ludwig *et al.*, 2003), but some groups do not agree about the correlation of mutation of this gene to the artemisinin (Dondorp *et al.*, 2009; Valderramos *et al.*, 2010).

Several strategies have been used to control malaria in the world, and these rely on the efficient and fast treatment of infected individuals, environmental measures including vector control programs and prevention by the stimulation of use of insecticide-treated bednets (WHO 2010b). Given the ever-looming surge of resistance of the parasite against the drugs currently in use, the development of an efficient vaccine or novel drugs are important issues.

The symptoms of malaria are linked to the stage of schizogony. After the invasion of erythrocyte the parasite consumes and destroys the intracellular proteins, especially hemoglobin which is polymerized into an inert substance denominated hemozoin or malarial pigment. According to classical symptomatology, fever coincides with lysis of red blood cells (RBCs) and is caused by the release into the bloodstream of the hemozoin and other toxic parasite products. Coincident with the rupture of erythrocyte occurs the increased expression of TNF- α and other cytokines (TNF- β and IL-6) which can also induce the release of other mediators (prostaglandin and TGF- β) that are responsible for the onset of symptoms (including fever) and tissue damage (Beeson & Brown 2002; Hemmer *et al.*, 2006).

P. falciparum is strongly associated with a potentially fatal form of the disease, most affecting young children, non-immune adults and pregnant women, although recent reports indicate an underestimation of the severity of *P. vivax* infections (Alexandre *et al.*, 2010). The pathogenesis of human *P. falciparum* infection is a complex interaction of parasite induced RBCs alterations (Maier *et al.*, 2009) and microcirculatory anomaly, (Grau *et al.*, 2003) accompanied by local and systemic immune reactions, resulting in a accumulation or sequestration of parasite infected RBCs in various organs, such as the brain, lung and placenta, and together with other factors is important in the pathogenesis of severe forms of malaria (Marsh *et al.*, 1995; Buffet *et al.*, 2011). RBC infected with early parasite stages (rings) display mild modifications of adhesion and/or deformability properties and may circulate, whereas late parasite stages, called trophozoites and schizonts (mature forms), have substantial alterations of adhesion and deformability that favor their sequestration in small vessels, thus preventing their circulation in the peripheral blood. Sequestration of mature forms is induced by their adherence to endothelial cells, blood cells, platelets, and uninfected RBCs. These interactions are mediated by multiple host receptors recognized by parasite adhesins (Maier *et al.*, 2009). Cerebral malaria is the main clinical manifestation of severe *P. falciparum* infection and seems to be responsible for most deaths. It is characterized by coma, often with convulsions (Lalloo *et al.*, 1996). Severe anaemia is probably the second most common presentation of severe *P. falciparum* infection and probably results from RBCs destruction, indirect destruction of parasitized erythrocytes by immune mechanisms and reduced erythropoiesis associated with imbalances in cytokine concentrations (Menendez *et al.*, 2000; Ekvall 2003). Renal dysfunction or failure, circulatory collapse and shock, disseminated intravascular coagulation and spontaneous bleeding, and acidosis can also occur. Among adults with malaria, pregnant women are particularly susceptible to malaria,

despite substantial immunity before pregnancy, and the risk is highest in first pregnancies. The major complications of infection are maternal anaemia, which in turn increases maternal deaths, and reduced infant birthweight from a combination of intrauterine growth retardation and premature delivery leading to excess infant mortality. In some settings maternal malaria may also cause spontaneous abortion or stillbirth (Granja *et al.*, 1998). Severe clinical forms are uncommon in infections with *P. vivax* however in countries where this parasite is dominating, more and more frequently is becoming common severe cases and even deaths to *P. vivax* infection are reported (Anstey *et al.*, 2009). In Brazil cases such as severe rhabdomyolysis (Siqueira *et al.*, 2010) and immune thrombocytopenic purpura have been reported (Lacerda *et al.*, 2004) and in south-east Asia, especially in India and Vietnam, cases of acute renal failure were documented (Sanghai & Shah 2010). A serious problem encountered in the *P. vivax* infection are hypnozoites, this liver stage that can cause relapses many months or even years after the initial infection, and these hypnozoites can only be eliminated by additional treatment with primaquine (Watkins & Sibley 2011).

2. The life cycle of plasmodium

Laveran was responsible for the discovery of the Plasmodium, observing them in human erythrocytes and was the first to describe it in 1880. The life cycle of parasites of humans Plasmodium genus is very similar between species, showing two distinct phases. The life cycle of malaria parasite is complex, and there are four critical points in the life cycle of *Plasmodium* parasites in which a small number of parasites rapidly multiply to generate much larger populations. These life cycle stages are male gamete development, sporozoite formation, liver stage development and blood stage asexual reproduction. The first two of these processes occur within the mosquito vector, and the second two processes take place in the vertebrate host.

Infective sporozoites from the salivary gland of the Anopheles mosquito are injected into the human host along with anticoagulant-containing saliva to ensure an even-flowing blood meal. Once entered in the human bloodstream, *P. falciparum* sporozoites reach the liver and penetrate the liver cells where they remain at for 9–16 days and undergo asexual replication known as exo-erythrocytic schizogony. Each sporozoite gives rise of thousands of merozoites inside the hepatocyte and each merozoite can invade a red blood cell (RBC) upon release from the liver. According to the Plasmodium species, the liver phase takes on average 6 days (*P. falciparum*), 10 days (*P. vivax*), or 15 days (*P. ovale* and *P. malariae*).

Merozoites enter erythrocytes by a complex invasion process, requiring a series of highly specific molecular interactions. Asexual division starts inside the erythrocyte and the parasites develop through different stages therein. The early trophozoite stage is often referred to as the “ring form”. Trophozoite enlargement is accompanied by highly active metabolism. The end of this stage is marked by multiple rounds of nuclear division without cytokinesis resulting in the formation of schizonts. Each mature schizont contains up to 32 merozoites and these are released after lysis of the RBC to invade further uninfected RBCs. This release coincides with the sharp increases in body temperature during the progression of the disease. This cycle takes about 36–48 h in *P. falciparum*, 48 h in *P. vivax* and *P. ovale* and 72 h in *P. malariae*. A small proportion of the merozoites in the RBCs eventually differentiate to produce micro and macrogametocytes. These gametocytes are essential for transmitting the infection to new hosts through female *Anopheles* mosquitoes.

A mosquito taking a blood meal on an infected individual may ingest these gametocytes into its midgut, where macrogametocytes form macrogametes and exflagellation of microgametocytes produces microgametes. These gametes fuse, undergo fertilization and form a zygote. This transforms into an ookinete, which penetrates the wall of a cell in the midgut and develops into an oocyst. Inside the oocyst many nuclear divisions occur, resulting in thousands of sporozoites and they migrate to the salivary glands for onward transmission into another host.

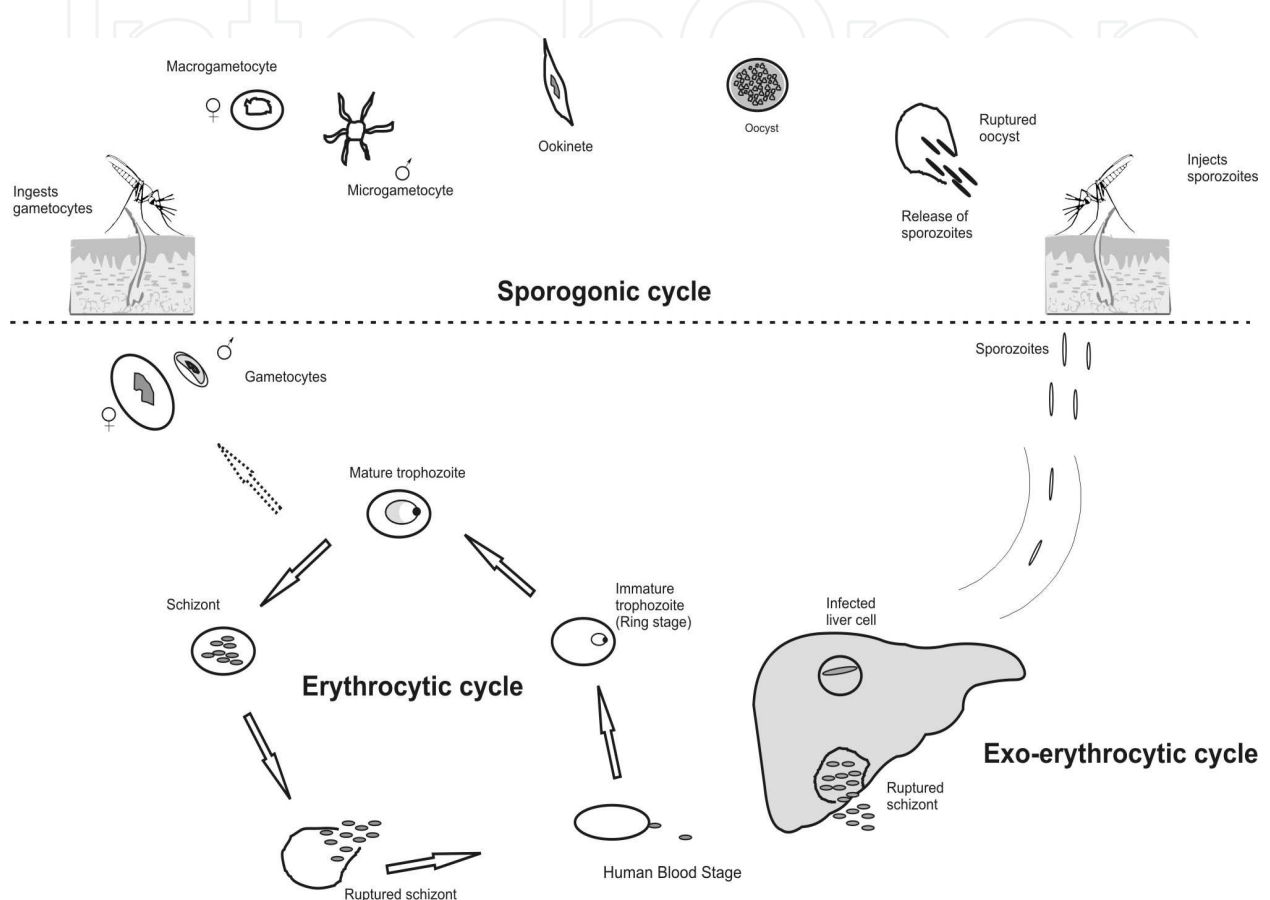


Fig. 2. During a blood meal, the mosquito inoculates sporozoites into the human host, infecting the liver cells initiating the exo-erythrocytic cycle. The parasites multiply, forming the schizonts, which then rupture releasing merozoites. The merozoites infect red blood cells, initiating the erythrocytic cycle (differentiating into ring, trophozoite and schizont); after the rupture of the red blood cells that contain them, merozoites are released and invade other red blood cells; some parasites differentiate into male (microgametocytes) and female (macrogametocytes) sexual forms the mosquitoes, by feeding off infected patients, ingest the gametocytes; the gametocytes evolve into gametes, and the microgametes penetrate the macrogametes generating zygotes, which in turn evolve into motile ookinetes; these invade the midgut wall of the mosquito, where they develop into oocysts; where sporogony takes place, releasing sporozoites; these migrate to the mosquito salivary gland.

The erythrocytic stages of the malaria parasite are responsible for the symptoms and pathology of the disease (Miller *et al.*, 1994). Immunity against the erythrocytic stages is not well understood, although they are important from the vaccine development perspective (Good 2001). To a greater or lesser extent, all four species have been cultured or maintained

in vitro; *P. falciparum*, however, is the only species for which all life cycle stages have been established in culture (Hollingdale 1992).

Cultivation of the intraerythrocytic stages of *P. falciparum* is vital for many aspects of malaria research. The *in vitro* continuous cultivation of the erythrocytic stages of *P. falciparum* achieved by (Trager & Jensen 1976) was a turning point in the history of malaria research. Since the establishment of this technique and refinements thereafter, the pace of research on malaria has increased significantly.

The methods for cultivation of the erythrocytic stages of *P. falciparum* reported have been usefully applied in nearly every aspect of research on malaria: chemotherapy, drug resistance, immunology and vaccine development, pathogenesis, gametocytogenesis and mosquito transmission, genetics, the basis for resistance of certain mutant red cells, cellular and molecular biology and biochemistry of the parasites and of their relationship with their host erythrocyte (Trager & Jensen 1997).

Although several methods have been developed, the technique used for the *in vitro* culture of the intraerythrocytic stages of *P. falciparum* remains essentially the same as that originally described by Trager and Jensen. The protocol described by these authors was based on the use of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered RPMI tissue culture medium supplemented with human serum, erythrocytes and sodium bicarbonate. Since then, efforts and time have been invested in trying to improve the *in vitro* growth of the asexual stages of the *Plasmodium* life cycle.

The availability of the technique has also spawned efforts to cultivate other malaria pathogens, both human and non-human. In most instances, it is more convenient working with malarial parasites from non-human hosts because they can be maintained *in vivo*, thereby allowing testing for infectivity of *in vitro*-cultured stages in the vertebrate host and providing an animal model for the study of the parasite in the human host, as the case culture malarial parasites infecting simian, avian, and rodent hosts.

Techniques for cultivation of the exoerythrocytic stages of avian malarial parasites *P. gallinaceum*, *P. lophurae*, and *P. fallax* were described by Huff (Huff 1964). Primary cultures of hepatocytes from rhesus monkeys (*Macaca mulatta*) were used to support growth of several simian malarias (*P. cynomolgi*, *P. knowlesi*, *P. coatneyi*, and *P. inui*) (Millet *et al.*, 1988). In a later study, the same group used rhesus hepatocytes for cultivation of developmental stages of *P. fieldi* and *P. simiovale*, two parasites that infect macaques (Millet *et al.*, 1994). Erythrocytic development of a number of simian malarial parasites has been studied, including that of *P. knowlesi*, *P. cynomolgi*, *P. fragile*, *P. gonderi*, *P. coatneyi*, *P. inui*, *P. fieldi*, and *P. simiovale*. Some of these aforementioned malarias are non human facsimiles of human malarias. Among the rodent malarias, the erythrocytic stages of *P. berghei* and *P. chabaudi* have been cultivated *in vitro* (Mons *et al.*, 1983; O'Donovan & Dalton 1993). The rodent malarial parasite *P. berghei* from *in vivo*-infected livers was maintained in primary cultures of rodent liver cells by Foley *et al.* (Foley *et al.*, 1978). Among the avian malarias, the erythrocytic stage of *P. lophurae* has been cultivated *in vitro* (Langreth & Trager 1973).

3. Characterization of Isoprenoid pathway by metabolic labeling

Widespread resistance to most anti-malarial drugs and the unavailability of an effective vaccine have fueled the emergence of malaria in recent years as a major global health and economic burden. Despite these hurdles, the field of malaria research has witnessed some extremely notable developments in the recent past including sequencing of the malaria

genome (Gardner *et al.*, 2002) the application of proteomics to studying malaria life cycle (Florens *et al.*, 2002), the malaria transcriptome (Bozdech *et al.*, 2003), and several web resources such as Mal-Vac (database of malaria vaccine candidates), PlasmoDB (genome database of the *Plasmodium* genus), and VarDB (database for antigenic variation genes families) (Chaudhuri *et al.*, 2008; Hayes *et al.*, 2008; Aurrecochea *et al.*, 2009).

A thorough knowledge of the biochemistry of *P. falciparum* is required in order to develop new drugs. This aim can be achieved by two means: either by focusing on validated targets in order to generate new drug candidates; or by identifying new potential targets for malaria chemotherapy. This last strategy will be commented and discussed in this chapter, focusing on the biosynthesis of isoprenoids. In the apicomplexan parasites of the genus *Plasmodium* the isoprenoid pathway is localized in the apicoplast which was acquired millions of years ago during an evolutionary event (Lim & McFadden 2010). At this occasion, the ancestor of the phylum apicomplexa gained a plastid by the secondary endosymbiosis of a photosynthetic eukaryote (McFadden *et al.*, 1996). The chloroplast of this photosynthetic eukaryote was retained and during evolution, many chloroplastid genes were transferred to the apicomplexan nucleus while others were lost (Funes *et al.*, 2002; Moore *et al.*, 2008). In the case of malaria parasites, especially its most virulent species *P. falciparum*, a series of new “plant-like” enzymes associated with this organelle were recently discovered (Luo *et al.*, 1999).

When testing for metabolic activities, usually the cultivable blood stage forms of *P. falciparum* are employed (Trager & Jensen 1976), that we will describe briefly below. Since the parasite is an obligate intracellular organism in this stage, several precautions must be taken to not confound host cell and parasite metabolic activities. Also, the chemical nature of an unlabeled or radiolabeled precursor plays an important role, since some substrates do not enter the red blood cell or do not cross the parasitophorous vacuole or the parasite cell membrane. Other substrates may require solvents which are toxic to either the parasite or the red blood cell. In the following, we describe the progress that was made in the detection of a number of metabolic activities of the parasites, many of which configure prime targets for drug intervention.

One of the strategies to identify each product of a metabolic pathway is the metabolic labeling using a radioactive specific precursor and a posterior analysis by an appropriate method. Due to the difficulty to obtain higher quantities of parasite biomass, the labeling with non-radioactive isotopes often does not lead to clear results, for this reason, most research groups rely on radiolabeled compounds when monitoring metabolic turnover. Several criteria must be evaluated before proceeding to experimental metabolic labeling of intraerythrocytic stages of *P. falciparum*: 1- determine type of study (structural or biosynthetic), 2- choose labeling protocol and select a radiolabeled precursor(s), 3- check incorporation of radioactivity into the parasite, 4- if the incorporation is sufficient for analysis, proceed with steady-state, pulse, pulse-chase or sequential transfer protocols for analysis of intermediates of the metabolic pathway to be studied. If incorporation is insufficient for analysis, it is recommended to try using more labeled precursor or decrease the level of unlabeled precursor in the culture medium. In the next step, it is advantageous to check effects on the parasite and molecule(s) of interest and effects on incorporation of labeled precursors. Afterwards, the optimal concentration of unlabeled precursor is selected and the experiment may proceed with steady-state, pulse, pulse-chase or sequential transfer protocols for the analysis of intermediates of the metabolic pathway that is studied.

For the characterization the composites isoprenics in parasite, *P. falciparum* clone 3D7 was cultured according to the protocol described by Trager and Jensen (Trager & Jensen 1976) where human sera was substituted by Albumax I (0.5%). Parasites were grown in tissue culture flasks (75 cm²) a 40 ml volume with a gas mixture of 5% CO₂, 5% O₂, and 90% N₂. In asynchronous cultures we can to obtain the ring, trophozoite or schizont stages. In synchronous cultures (~ 15% parasitemia), the stage initially was in ring stage (1-20 h after reinvasion) after two treatments with 5% (w/v) D-sorbitol solution in water, for subsequent maintenance in culture until the differentiation to trophozoite (20-30 h after reinvasion) or schizont (30-35 h after reinvasion) stages. Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears.

Cultures of *P. falciparum* with a parasitemia of approximately 10% were labeled with the different precursors in normal RPMI 1640 medium for 15 h and after each stage purified. The asynchronous parasites were purified on a 40%/70%/80% discontinuous Percoll[®] gradient (Braun-Breton *et al.*, 1986). The culture was centrifuged at 2000 rpm for 5 min at room temperature, the pellet resuspended in RPMI-1640 (1:1 vol/vol), and carefully placed on top of the gradient. The tubes were centrifuged at 10,000 x g for 20 min at 20°C. The cells containing schizonts, trophozoite and ring stages, respectively were collected, washed twice in RPMI-1640, the pellet of parasites were stored in liquid N₂ for subsequent analysis. The other hand, synchronous culture with schizont stages predominantly were purified with magnetic column separation (MACS Separation Columns "CS"). The pre-equilibration, washing, and elution the column were all carried out at room temperature with RPMI-1640. The culture was centrifuged, the pellet resuspends in RMPI-1640 (1:10 vol/vol), 10 ml of the 10% suspension of erythrocytes were applied to a CS column assembled in a magnetic unit to remove non-infected erythrocytes, ring-infected erythrocytes, and young trophozoites. After wash the column by filling from top with 50 ml of RMPI-1640, the column was removed from the magnetic field and its contents eluted with 50 ml of RMPI-1640, thus, the schizonts stages fraction were obtained, centrifuged at 2,000 rpm for 5 min at room temperature, the supernatant discarded, and the pellet of parasites were stored in liquid N₂ for subsequent analysis. Parasite form was monitored by microscopic evaluation of Giemsa-stained thin smears.

In the following, we specifically focus i) on the methylerythritol phosphate (MEP) pathway which leads to the isoprenoids isopentenyl pyrophosphate (IPP) /dimethylallyl pyrophosphate (DMAPP) and ii) on downstream reactions which result in dolichol, ubiquinone, menaquinone, tocopherol, carotenoids and other related compounds.

4. Mevalonate-independent methylerythritol phosphate (MEP) pathway in *P. falciparum*

The MEP pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate, which yields 1-deoxy-D-xylulose-5-phosphate (DOXP) as a key metabolite (Rohmer *et al.*, 1993). Cassera and colleagues showed by metabolic labeling that the MEP pathway is functionally active in the intraerythrocytic stages of *P. falciparum*. Using different radioactive precursors such as [1-¹⁴C]sodium acetate, D-[U-¹⁴C]glucose and [2-¹⁴C]deoxy-D-xylulose, five intermediates of the MEP pathway were identified. The intermediates were isolated by high performance liquid chromatography (HPLC) and characterized by electrospray mass spectrometric analyses. All but one of the intermediates of the MEP pathway were characterized, including 1-deoxy-D-xylulose-5-phosphate, 2-C-methyl-D-erythritol-4-

phosphate, 4-(cytidine-5-diphospho)-2-C-methyl-D-erythritol, 4-(cytidine-5-diphospho)-2-C-methyl-D-erythritol-2-phosphate, and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate. The effect of fosmidomycin on the levels of MEP pathway intermediates was found to be most prominent in ring stages followed by schizont stages of *P. falciparum*. It was also shown that the MEP pathway provides IPP precursors for the synthesis of higher isoprenic compounds like ubiquinone and dolichol, and this was demonstrated by the decrease in the ubiquinone and dolichol content in fosmidomycin-treated parasites (Cassera *et al.*, 2004). To achieve efficient labeling, [1-¹⁴C] sodium acetate and D-[U-¹⁴C] glucose were employed instead of pyruvic acid, which is not incorporated by blood stage *P. falciparum*. These relatively simple compounds which enter in many biochemical pathways obviously require a refined analysis of synthesized molecules and this was achieved by demonstrating the chemical nature of the found molecules by different analytic methods.

5. Detection of N-linked glycoproteins in *P. falciparum*

Glycoconjugates have been shown to be important in the penetration, cellular growth, host immunity regulation and differentiation (Schwarz & Datema 1982). The presence of glycoproteins in *P. falciparum* has been demonstrated in several studies but remains controversial because there is little information addressing the function of *P. falciparum* glycoconjugated molecules. Also, the few available studies are inconsistent due to differing methodologies. N-linked glycosylation is a protein modification that occurs co-translationally in the endoplasmic reticulum. Kimura and colleagues described briefly the methodology used for detection of N-linked glycoproteins. For this purpose parasite culture containing ring stage parasites were labeled for 48 hs with D-[U-¹⁴C]glucose or D-[U-¹⁴C]mannose. The metabolic labeling was also done in the presence of 12 μM tunicamycin, the classic inhibitor of N-glycosylation. The total profile of glycoproteins was analyzed by SDS-PAGE of treated and untreated parasites. The N-linked glycoproteins were confirmed by 18 h radiolabeling with L-[³⁵S]methionine and affinity chromatography using Concanavalin A-Sepharose. In parallel, tunicamycin pre-treated parasites showed a differing band pattern, thus identifying protein species which carried N-glycosyl moieties. In order to increase L-[³⁵S]methionine or D-[U-¹⁴C] glucose labeling, the cultures were starved in methionine- or glucose-deficient medium for 1 h before the addition of the radioactive substance (Kimura *et al.*, 1996). By this approach, these authors identified N-linked glycoprotein when they used the radioactive precursor D-[U-¹⁴C]glucose. As above, although this form of labeling is unspecific, other methodologies confirmed the nature the compounds where radioactive glucose became incorporated. On the other hand, Dieckmann-Schuppert *et al.* (1992) did not detect N-linked glycoproteins using the specific 2-[³H]mannose, 6-[³H]glucosamine, or 1-[¹⁴C]mannosamine. In 1997 Gowda and colleagues confirmed that *P. falciparum* contains low levels of N-glycosylation activity. The amount of N-linked carbohydrates in whole parasite proteins is approximately 6% compared with the GPI anchors attached to proteins based on radioactive GlcN incorporated into the proteins (Gowda *et al.*, 1997). Bushkin *et al.* (2010) suggested that the occupation of N-glycan sites is markedly reduced in apicoplast proteins versus some secreted proteins in *Plasmodium*. Clearly, more work has to be done in order to elucidate the nature and purpose of N-linked glycoproteins in *Plasmodium*. Also, the differences in the results published by distinct groups mentioned above may have occurred due to differences in basic aspects of each experiment, such as the duration of labeling, the tracing compound used and the parasite stage that was analyzed.

6. Characterization of dolichol in *P. falciparum*

The protein modification by N-linked glycosylation is dependent on the *de novo* synthesis of dolichyl-P, a long chain non-sterol isoprene which acts as a membrane-bound carrier of oligosaccharides in the assembly of glycoproteins (Leloir 1977). Couto *et al* (1999) demonstrated the presence of dolichol, dolichyl-P and dolichyl-PP species of 11 and 12 isoprenic units in parasites of *P. falciparum* cultivated *in vitro*.

In these experiments, cultures of *P. falciparum* were labeled for 15 h with [1-(n)-³H]geranylgeranyl pyrophosphate triammonium salt ([1-(n)-³H]GGPP) or with [1-(n)-³H]farnesyl pyrophosphate triammonium salt ([1-(n)-³H]FPP). Each stage was purified by Percoll gradient; the pellets were extracted and analyzed by C18 reverse-phase HPLC (RP-HPLC). From extracts labeled with [1-(n)-³H]GGPP, 3 major peaks were detected: at 8 min, coincident with an authentic sample of geraniol, at 21 min, coincident with a dolichol standard with 11 isoprenic units and at 23 min, coincident with a dodecaprenol standard. Parasites labeled with [1-(n)-³H]FPP showed a number of other labeled products, but the presence of dolichol of 11 and 12 isoprenic units was evident (Couto *et al.*, 1999).

7. Characterization of ubiquinone and carotenoids in *P. falciparum*

Coenzyme Q is a molecule composed of a benzoquinone ring with a side chain of several isoprenic units, and the number of which defines the type of coenzyme Q. A polyprenyl diphosphate synthase is involved in the elongation of the side chain (Ogura *et al.*, 1997). This isoprenic chain is then attached to p-hydroxybenzoic acid (PHBA), which is synthesized via the shikimate pathway. The isoprenic chain then allows the molecule to attach to the inner membrane of mitochondria, where it participates in many metabolic processes, like the electron transport chain (Ernster & Dallner 1995).

Macedo *et al.* (2002) had identified coenzyme Q8 and coenzyme Q9 by metabolic labeling of parasites with [1-¹⁴C]acetic acid, [1-¹⁴C]isopentenyl pyrophosphate triammonium salt ([1-¹⁴C]IPP), [1-(n)-³H]FPP, and [1-(n)-³H]GGPP in all intraerythrocytic stages. To our knowledge, this was the first report on the incorporation of [1-¹⁴C]IPP into *P. falciparum*. It is well known that the considerable increase in total lipid content associated with *P. falciparum* invasion is due to the existence of an intense lipid transport system in infected erythrocytes. The efficient uptake of [1-(n)-³H]FPP, [1-(n)-³H]GGPP and, to a lesser extent, [1-¹⁴C]IPP, may be ascribed to this transport mechanism for lipid-like components. The parasite is capable of synthesizing two different homologues of coenzyme Q, depending on the given radioactive intermediate. When labeling is performed with [1-(n)-³H]FPP, coenzyme Q with an isoprenic chain of 40 carbons (Q8) is detected; while [1-(n)-³H]GGPP labeling leads to Q9 (45 carbons) chains. These findings can be explained by the fact that both FPP and GGPP are substrates of the prenyltransferase involved in the biosynthetic pathway of the isoprenic chain of ubiquinone as shown in other systems (Ogura & Koyama 1998). This difference in the length of the isoprenic chain according to the precursor was also observed in the biosynthesis of dolichols in *P. falciparum*. In order to check whether this difference could be induced by the length of the isoprenoid intermediate, the basic isoprenic unit [1-¹⁴C]IPP was used as a metabolic marker. It would be expected that by labeling with [1-¹⁴C]IPP, both coenzymes Q would be detected. Surprisingly, HPLC analysis showed a single radioactive peak, which co-eluted with a coenzyme Q9 standard. The enzyme of *P. falciparum* (www.PlasmoDB.org, entry PfB0130w) that biosynthesizes the isoprenic chain attached to

benzoquinone ring of coenzyme Q was cloned and expressed and showed octaprenyl pyrophosphate synthase activity. Enzymatic activity was measured by determination of the amount of [1-¹⁴C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The recombinant and native versions of the enzyme had similar Michaelis constants with the substrates [1-¹⁴C]IPP and farnesyl pyrophosphate. The initial rate was calculated by determining the quantities of product formed or IPP consumed at each time point by counting the ¹⁴C radioactivity in the butanol phase (product) and in the aqueous phase (IPP). The recombinant protein, as well as *P. falciparum* extracts, showed an octaprenyl pyrophosphate synthase activity, with the formation of a polyisoprenoid with eight isoprenic units, as detected by reverse-phase HPLC and reverse-phase TLC, and confirmed by electrospray ionization and tandem MS analysis. Additionally, the recombinant enzyme could be competitively inhibited in the presence of the terpene nerolidol. Since the *P. falciparum* enzyme shows quite low similarity to its human counterpart, decaprenyl pyrophosphate synthase, it was suggested that the identified enzyme and its recombinant version could be exploited in the screening of novel drugs (Tonhosolo *et al.*, 2005). The enzyme octaprenyl pyrophosphate synthase (PfB0130w) turned out to be a bi-functional enzyme with phytoene synthase activity, which was shown by *in vitro* enzymatic assays using [1-(n)-³H]GGPP as a substrate, followed by HPLC analysis and confirmation by LC-APCI-MS/MS analysis. The identification of the enzyme phytoene synthase showed that intraerythrocytic stages of *P. falciparum* can perform the crucial step of the pathway that leads to the biosynthesis of carotenoids. Carotenoids are widespread lipophilic pigments synthesized by all photosynthetic organisms and some nonphotosynthetic fungi and bacteria. All carotenoids are derived from the C40 isoprenoid precursor geranylgeranyl pyrophosphate. HPLC analysis from extracts of intraerythrocytic stages of *P. falciparum* labeled with [1-(n)-³H]GGPP, revealed several compounds in all intraerythrocytic stages, with retention times coincident with lutein, phytoene, phytofluene, *all-trans*- β -carotene, neurosporene and 6-*all-trans*-lycopene. Some of these compounds were structurally characterized by electrospray mass spectrometric analysis (Tonhosolo *et al.*, 2009).

Considering that carotenoid biosynthesis is absent in humans, and also that possibly other uncharacterized carotenoid synthesizing enzymes are present, in *Plasmodium*, it is possible to speculate that this pathway could be exploited for the design of new antimalarial drugs. Indeed, sequence data from additional organisms, functional studies, improved bioinformatics screening approaches, together with biochemical evidence, may reveal whether other interesting targets and pathways are present in the phylum Apicomplexa.

8. Vitamin E biosynthesis

The biosynthesis of vitamin E depends on both the MEP and shikimate pathways. This compound consists of a polar chromanol head group attached to a hydrophobic phytyl (tocopherols) or geranylgeranyl (tocotrienols) tail, both of which are critical for their roles as lipid-soluble antioxidants (Schneider 2005).

Using [1-(n)-³H]GGPP or [1-(n)-³H]FPP as radiotracer precursors and HPLC systems for purification of vitamin E, the biosynthesis of α and γ -tocopherol was detected in the three intraerythrocytic stages from 5×10^8 parasites. To confirm the chemical identity of these compounds, unlabeled extracts from 10^{10} parasites were purified by HPLC, the retention time of tocopherol isomers was collected and analyzed by gas chromatography coupled to a

mass spectrometer (GC-MS). Although the two isomers were found in parasites as well as in erythrocytes and culture medium extracts, the parasite extracts showed higher concentrations than the other samples (Sussmann 2010).

Usnic acid is a secondary metabolite from lichens and capable to inhibit the 4-hydroxyphenylpyruvate dioxygenase, an enzyme from the shikimate pathway which is responsible for the biosynthesis of homogentisate from hydroxyphenylpyruvate. Homogentisate is the aromatic portion which receives the isoprenoid side chain from MEP pathway to form vitamin E isomers. When labeled parasites are treated with 25 μM of usnic acid, the biosynthesis of α and γ -tocopherol was inhibited in $53,5 \pm 7\%$ (Sussmann 2010).

9. Menaquinone biosynthesis

In plants and cyanobacteria the shikimate pathway and MEP provide the precursors for the biosynthesis of phyloquinone (PhQ) while in bacteria menaquinone is synthesized (Lombardo *et al.*, 2006). Tonhosolo *et al.* (2010) showed by metabolic labeling with the precursor [1-(n)- ^3H]GGPP that intraerythrocytic stages of *P. falciparum* biosynthesizes menaquinone (MQ-4), employing different chromatographic methods reported for this type of molecule and further confirmed the nature of the molecules by ESI-MS/MS analysis. Additionally, they showed that the mycobacterial inhibitor of menaquinone synthesis Ro 48-8071 also suppressed MQ biosynthesis and growth of parasites, pointing possibly again to an interesting drug target.

10. Posttranslational modification

10.1 Protein farnesylation and geranylgeranylation in *P. falciparum*

Post-translational modification of proteins with isoprenoids was first recognized as a general phenomenon in 1984 (Schmidt *et al.*, 1984). The isoprenyl group is linked post-translationally to cysteine residues at the C-terminus of the protein through a thioether bond (McTaggart 2006). Studies have shown that FPP (15 carbons) and GGPP (20 carbons) are the most common isoprenoids found attached to proteins. Several of the proteins that undergo these modifications have been identified and may participate in important cell regulatory functions, particularly signal transduction pathways (Zhang & Casey 1996). Protein prenylation is a general phenomenon in eukaryotic cells and has also been described for several protozoan parasites (Lujan *et al.*, 1995; Field *et al.*, 1996; Shen *et al.*, 1996; Ibrahim *et al.*, 2001) including *P. falciparum* (Chakrabarti *et al.*, 2002).

In order to investigate the presence of isoprenylated proteins in *P. falciparum*, the labeled intermediates [1-(n)- ^3H]FPP and [1-(n)- ^3H]GGPP were used. Parasites were incubated with radioactive for 18 h, purified on a Percoll gradient, lysed, and analyzed by SDS/PAGE and autoradiography. [1-(n)- ^3H]GGPP labeled proteins appeared in the ring, trophozoite, and schizont stages. Non-infected red blood cells showed no incorporation of radioactivity under these conditions. The isoprenylated proteins were later identified as members of the Ras and Rab protein family (Rodrigues Goulart *et al.*, 2004).

Moura *et al.* (2001) and Rodrigues Goulart *et al.* (2004) showed that terpenes can inhibit protein isoprenylation in *P. falciparum*. The process of protein prenylation is a very attractive target for the development of new drugs for cancer and parasites (Docampo & Moreno 2001; Stresing *et al.*, 2007). One of the most potent bisphosphonates clinically used

to treat bone resorption diseases, risedronate, inhibited the protein isoprenylation in *P. falciparum* (Jordao *et al.*, 2011). In order to investigate the mechanism of action for risedronate in intraerythrocytic stages of *P. falciparum*, parasites were incubated with or without risedronate and with [1-(n)-³H]FPP, [1-(n)-³H]GGPP and [¹⁴C] IPP, purified on a Percoll gradient, lysed, and analyzed by TLC and SDS-PAGE. The results showed that protein prenylation is inhibited by decreasing the biosynthesis of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Additionally, it was demonstrated that risedronate inhibits the transference of [1-(n)-³H]FPP to proteins but not the [1-(n)-³H]GGPP in *P. falciparum*.

10.2 Protein dolichylation in *P. falciparum*

Another type of protein modification is the attachment of a dolichyl group to proteins. This type of modification is characterized by covalently and post-translationally bound dolichyl groups to the C-terminal cysteine residues of proteins. Protein dolichylation was described in tumor cells and dolichylated proteins could be involved in the cell cycle control (Hjertman *et al.*, 1997).

Our group has previously shown that *P. falciparum* synthesizes dolichols of 11 and 12 isoprene units (Couto *et al.*, 1999) and that these compounds can be attached to a group of 21–24 kDa proteins of this parasite (Moura *et al.*, 2001). To confirm the existence of dolichyl groups attached to *P. falciparum* proteins, D'Alexandri *et al.* (2006) performed *in vitro* metabolic labeling of the parasites with [1-(n)-³H]FPP or [1-(n)-³H]GGPP. They used these precursors instead of [³H]dolichol for analysis of protein dolichylation because commercially available [³H]dolichol has dolichols of 16 and 21 isoprene units that are longer than those synthesized by *P. falciparum*. After metabolic labeling, the proteins were extracted, extensively delipidated and analyzed by SDS-PAGE. RP-TLC and RP-HPLC analysis of [1-(n)-³H]FPP-labeled compounds released from the 21 to 28 kDa from *P. falciparum* proteins revealed that a dolichol of 11 isoprene units and a polyisoprenoid of 12 isoprene units can be attached to proteins of this parasite. The dolichol structure was confirmed by electrospray-ionization mass spectrometry analysis. Treatment with protein synthesis inhibitors and RP-HPLC analysis of the proteolytic digestion products from parasite proteins labeled with [³⁵S]cysteine and [1-(n)-³H]FPP showed that the attachment of dolichol to protein is a post-translational event and probably occurs via a covalent bond to cysteine residues. This was the first demonstration of protein dolichylation in parasites, and also may represent a new potential target for anti-malarial drugs.

11. Concluding remarks

The use of radioactive tracers in metabolic labeling in cultures of *P. falciparum* allowed the identification of many steps of the isoprenoid biosynthesis. In figure 3 we summarize the intermediaries' biosynthesizing by the MEP pathway and the isoprenoids identified in *P. falciparum* until the moment. This pathway is different from the human host and we hypothesize that the identification of related enzymes may directly lead to the development of new antimalarial drugs. In many aspects, radiotracers are important, often indispensable tools for the identification of metabolic intermediates not only in *Plasmodium* but also in many other intracellular protozoa and may ultimately help to point to yet undetected novel drug targets.

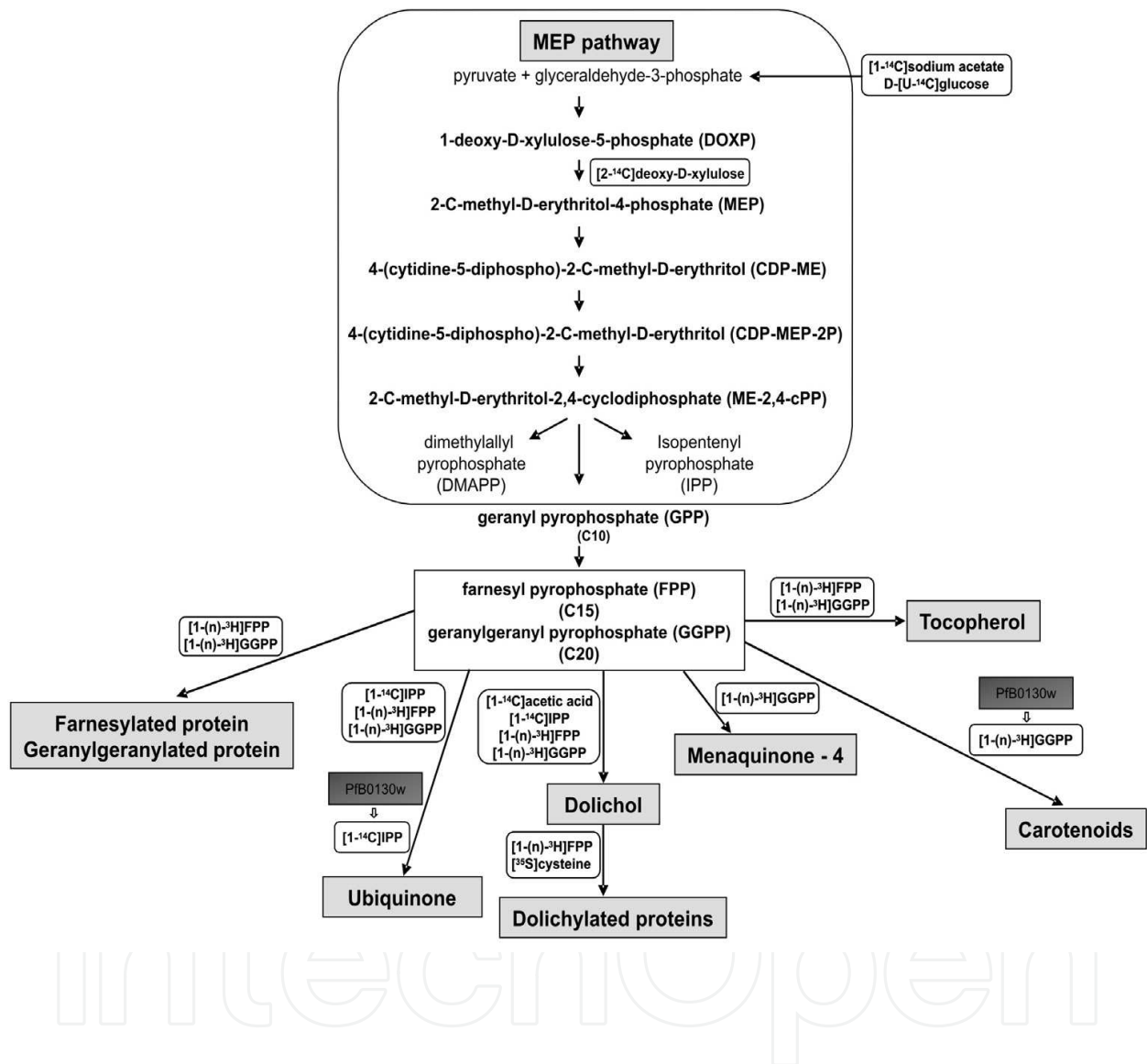


Fig. 3. Isoprenoids biosynthesized by *P. falciparum*. IPP and DMAPP are biosynthesized by the 2-C-methyl-D-erythritol (MEP)-4-phosphate pathway. Shaded boxes indicate presence the isoprenoids biosynthesized by malaria parasite identified until this moment. White boxes indicate radioactive tracers used for identification of products biosynthesized by *P. falciparum*. PfBO130w correspond to a bi-functional enzyme octaprenyl pyrophosphate synthase/phytoene synthase that use [1-¹⁴C]IPP or [1-(n)-³H]GGPP respectively as substrate.

12. Acknowledgments

This work was supported by grants from Brazilian Agencies CNPq and FAPESP (Brazil). F.M.J. and R.A.C.S. are the recipient of a post-graduate fellowship from CNPq. A.Y.S. and H.B.G. receive post-graduate fellowships from FAPESP. R.T. is the recipient of a post-doctoral fellowship from PNPd/CAPES.

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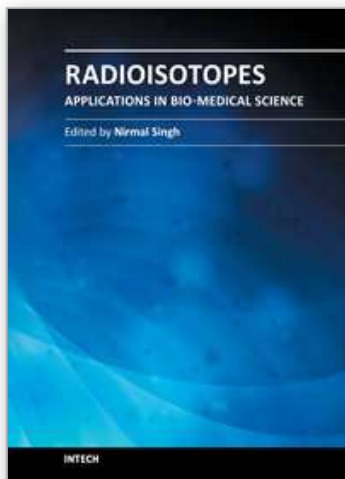
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Radioisotopes - Applications in Bio-Medical Science

Edited by Prof. Nirmal Singh

ISBN 978-953-307-748-2

Hard cover, 320 pages

Publisher InTech

Published online 21, November, 2011

Published in print edition November, 2011

The book *Radioisotopes - Applications in Bio-Medical Science* contains two sections: *Radioisotopes and Radiations in Bioscience* and *Radioisotopes and Radiology in Medical Science*. Section I includes chapters on medical radioisotope production, radio-labeled nano-particles, radioisotopes and nano-medicine, use of radiations in insects, drug research, medical radioisotopes and use of radioisotopes in interdisciplinary fields etc. In Section II, chapters related to production of metal PET (positron emission tomography) radioisotopes, 3-dimensional and CT (computed tomography) scan, SS nuclear medicine in imaging, cancer diagnose and treatments have been included. The subject matter will be highly useful to the medical and paramedical staff in hospitals, as well as researchers and scholars in the field of nuclear medicine medical physics and nuclear bio-chemistry etc.

How to reference

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Emilia A. Kimura, Gerhard Wunderlich, Fabiana M. Jordão, Renata Tonhosolo, Heloisa B. Gabriel, Rodrigo A. C. Sussmann, Alexandre Y. Saito and Alejandro M. Katzin (2011). Use of Radioactive Precursors for Biochemical Characterization the Biosynthesis of Isoprenoids in Intraerythrocytic Stages of *Plasmodium falciparum*, *Radioisotopes - Applications in Bio-Medical Science*, Prof. Nirmal Singh (Ed.), ISBN: 978-953-307-748-2, InTech, Available from: <http://www.intechopen.com/books/radioisotopes-applications-in-bio-medical-science/use-of-radioactive-precursors-for-biochemical-characterization-the-biosynthesis-of-isoprenoids-in-in>

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