Effect of fosmidomycin on metabolic and transcript profiles of the methylerythritol phosphate pathway in *Plasmodium falciparum*

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In Plasmodium falciparum, *the formation of isopentenyl diphosphate and dimethylallyl diphosphate, central intermediates in the biosynthesis of isoprenoids, occurs via the methylerythritol phosphate (MEP) pathway. Fosmidomycin is a specific inhibitor of the second enzyme of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase. We analyzed the effect of fosmidomycin on the levels of each intermediate and its metabolic requirement for the isoprenoid biosynthesis, such as dolichols and ubiquinones, throughout the intraerythrocytic cycle of* P. falciparum. *The steady-state RNA levels of the MEP pathway-associated genes were quantified by real-time polymerase chain reaction and correlated with the related metabolite levels. Our results indicate that MEP pathway metabolite peak precede maximum transcript abundance during the intraerythrocytic cycle. Fosmidomycin-treatment resulted in a decrease of the intermediate levels in the MEP pathway as well as in ubiquinone and dolichol biosynthesis. The MEP pathway associated transcripts were modestly altered by the drug, indicating that the parasite is not strongly responsive at the transcriptional level. This is the first study that compares the effect of fosmidomycin on the metabolic and transcript profiles in* P. falciparum, *which has only the MEP pathway for isoprenoid biosynthesis.*

Key words: *Plasmodium falciparum* - malaria - fosmidomycin - isoprenoid biosynthesis - real time polymerase chain reaction

Malaria is a leading cause of morbidity and mortality in the tropical regions, with 300 to 500 million clinical cases and 1.5 to 2.7 million deaths per year (Snow et al. 2005). With the availability of the complete genome sequence from *Plasmodium falciparum*, increasing attention has focused on transcript profiling and proteomic analyses of the parasite stages responsible for severe disease and pathogenesis in humans (Florens et al. 2002, Bozdech et al. 2003, Le Roch et al. 2003, Nirmalan et al. 2004, Llinas et al. 2006).

Two different biosynthetic routes are used to produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) for isoprenoid biosynthesis including ubiquinones, dolichols and the prenylation of proteins (Sacchettini & Poulter 1997, Sinensky 2000, Barkovich & Liao 2001). In mammals, plants (cytoplasm), fungi, some bacteria and several protozoa, the isoprenic units are derived from the classical mevalonate pathway (Goldstein & Brown 1990). In plastids of plants, several algae, eubacteria, cyanobacteria and apicomplexa (apicoplast), the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway produces IPP and DMAPP (Rohmer 1999). The MEP pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate (GAP), which yields 1-deoxy-D-xylulose-5-phosphate (DOXP) catalyzed by DOXP synthase (DXS); for reviews see references (Lichtenthaler 1999, Rohmer 1999, Eisenreich et al. 2004, Rodriguez-Concepcion 2004). DOXP reductoisomerase (DXR) then catalyzes the intramolecular rearrangement and reduction of DOXP to MEP. The activity of this enzyme is specifically inhibited by fosmidomycin (Kuzuyama et al. 1998). Several subsequent reaction steps are necessary for the conversion of MEP to IPP and DMAPP (Fig. 1).

Discovery of the MEP pathway for isoprenoid biosynthesis in *P. falciparum* revealed several antimalarial drug targets (Jomaa et al. 1999). Jomaa and co-workers demonstrated that fosmidomycin and its derivate FR900098, are able to inhibit the growth of *P. falciparum* in culture and to cure mice infected with the related malaria parasite, *P. vinckei* (Jomaa et al. 1999). Recent field trials in humans have also demonstrated the effectiveness of fosmidomycin in the treatment of human malarial infections, but it has to be administered for more than four days when used alone (Missinou et al. 2002, Borrmann et al. 2005, 2006). Recently, biochemical and mass spectrometric analyses revealed that the MEP pathway is functionally active in all intraerythrocytic stages of *P. falciparum* (Cassera et al. 2004).

In this study we characterized the effect of fosmidomycin on the metabolic levels of each intermediate of the MEP pathway as well as dolichol and ubiquino-

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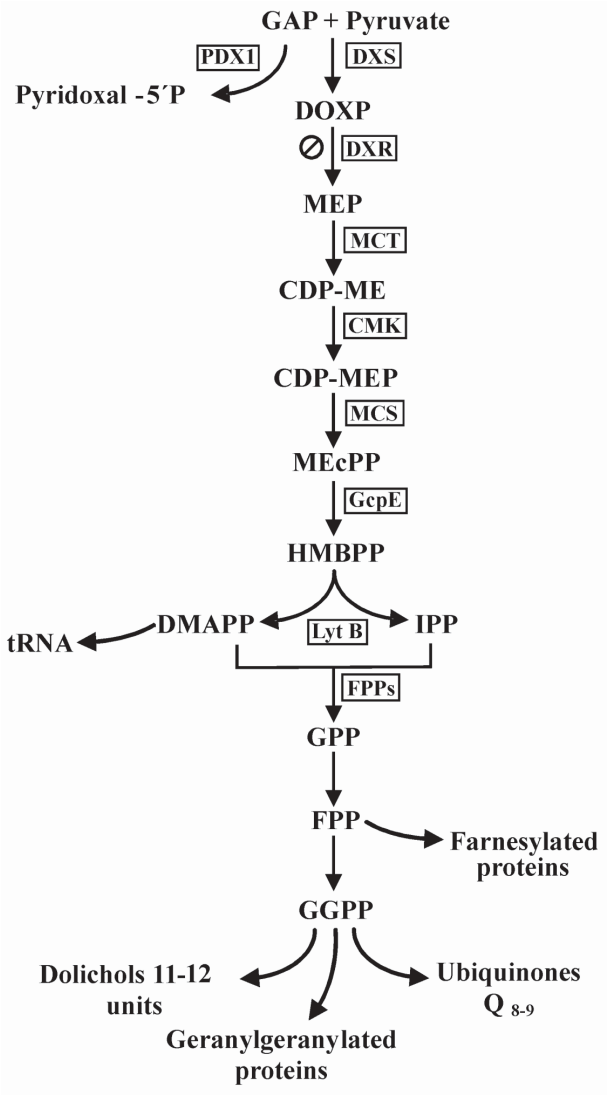

Fig. 1: isoprenoid biosynthesis pathway in *Plasmodium falciparum*. ∅ indicates the step that is inhibited by fosmidomycin. The following metabolites and enzymes are shown: PDX1, pyridoxal-5´-phosphate synthase; GAP, glyceraldehyde-3-phosphate; DOXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DOXP synthase; MEP, 2C-methyl-D-erythritol-4-phosphate; DXR, DOXP reductoisomerase; CDP-ME, 4-(cytidine-5-diphospho)-2C-methyl-D-erythritol; MCT, 2C-methyl-D-erythritol-4-(cytidine-5-diphospho) transferase; CDP-MEP, 4-(cytidine-5-diphospho)-2C-methyl-D-erythritol-2-phosphate; CMK, CDP-ME kinase; MEcPP, 2C-methyl-D-erythritol-2,4-cyclodiphosphate; MCS, MEcPP synthase; HMBPP, 4-hydroxy-3-methyl-but-2-enyl pyrophosphate; GcpE, HMBPP synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; LytB, HMBPP reductase; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FPPs, FPP synthase; GGPP, geranylgeranyl diphosphate.

nes in ring, trophozoite and schizont stages of *P. falciparum* parasites and correlated these to the steady-state MEP enzyme transcript levels under drug pressure.

MATERIALS AND METHODS

Experimental design - Three characteristic developmental stages: ring (6 h ± 4 after reinvasion), trophozoite (23 h ± 4 after reinvasion) and schizont (36 h ± 4 after reinvasion) were chosen as representative for the intraerythrocytic cycle of *P. falciparum*. Two independent experiments using [1-^{14}C]sodium acetate as a metabolic precursor were conducted to monitor each intermediate of the MEP pathway, dolichols and ubiquinones for each developmental stage. [1-^{14}C]sodium acetate is well incorporated into the MEP pathway intermediates in *P. falciparum* (Cassera et al. 2004) instead of [1-^{14}C]pyruvic acid or [2-^{14}C]pyruvic acid, which is not incorporated by blood-stage *P. falciparum* (Cranmer et al. 1995, Elliott et al. 2001). Two cycles after sorbitol synchronization, cultures in ring, trophozoite or schizont stages with approximately 10% parasitemia, untreated or treated with 1 µM fosmidomycin for 31 h, were labeled with 6.25 µCi/ml of [1-^{14}C]sodium acetate (56 mCi/mmol, Amersham Biosciences) in the last 17 h, and recovered for analysis. Seventeen hours of exposure time is the minimum time to detect ^{14}C incorporation into the MEP pathway intermediates (Cassera et al. 2004). The parasites were isolated by treatment with 0.1% (w/v) saponin for 5 min, followed by two washes with phosphate-buffered saline (PBS), pH 7.2, and stored in liquid N_2 for subsequent HPLC analysis. Analyses of metabolites were accomplished by using 1×10^{11} parasites of each stage obtained from treated or untreated synchronous cultures of *P. falciparum*. The same numbers of uninfected erythrocyte were analyzed in parallel. For transcript analysis, samples of RNA from three independent experiments were prepared using the same scheme of synchronization and treatment for the metabolic labeling described above. The IC_{50} value of fosmidomycin for the 3D7 strain is 1.25 ± 0.05 µM (Cassera et al. 2004). All experiments were performed with 1 µM fosmidomycin during 48 h because under these conditions only a small percentage of parasites die and the length of the developmental cycle are not affected. The overall protein synthesis was controlled by quantification of TCA precipitates and by SDS-PAGE analysis at 48 h. Protein synthesis was not affected in accordance to our earlier results (Cassera et al. 2004). Microscopic evaluation of Giemsa-stained thin smears was carried out very carefully to ensure that the parasites were at the same stage of development in control and fosmidomycin-treated cultures. Percent effect was determined as follows: [100 - (cpm or copy number in treated parasites × 100/cpm or copy number in untreated parasites)].

Parasite cultures - All experiments were performed with the *P. falciparum* 3D7 clone. Parasites were cultivated according to the method of Trager and Jensen (1976), modified by Kimura et al. (1996). Culture synchronization was carried out by two treatments with 5% (w/v) sorbitol solution in water (Lambros & Vanderberg 1979).

RNA isolation, cDNA synthesis and real-time quantitative transcript analysis - Total RNA was prepared directly from 200 µl of a saponin-treated cell pellet lysed in 2 ml Trizol® (Invitrogen) and RNA was extracted according to the manufacturer's instructions. Subsequent cDNA synthesis for construction of the standard curves and quantitative real time PCR were performed with 5 µg total RNA from ring, trophozoite or schizont stages

parasites, from three independent experiments untreated or treated with 1 μM fosmidomycin for 48 h. All RNAs were treated with DNAseI (RNAse free, Invitrogen) before cDNA synthesis, according to the manufacturer's instructions. First strand cDNA was then synthesized using Superscript II reverse transcriptase (Invitrogen) and a gene-specific primer mix with 2 pmol of each antisense oligonucleotide as described by the manufacturer. Gene-specific oligonucleotide primers for *P. falciparum* genes *DXS, DXR, MCT, CMK, MCS, GcpE, LytB, FPPs, PDX1*, and *liver stage antigen-1* (*Lsa-1*, negative control) were designed using the Primer Express Software v.1.5 (Applied Biosystems) (see Table I). PCR products from cDNA of each gene were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced. The purified plasmids were quantified and linearized with *Pst*I (Invitrogen). A representative standard curve was constructed for each gene using serial dilutions of the respective plasmids. For real-time PCR quantification of each generated cDNA, SYBR® green system was used (SYBR® green PCR Master Mix, Applied Biosystems) on a GeneAmp® 5700 Sequence Detection system (Applied Biosystems) according to the manufacturer's instructions. Experimental PCR conditions included 300 nM of each primer and cDNA corresponding to 100 ng converted RNA in 25 μl final volume per reaction. Each point was measured in triplicate. The real-time PCR consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification specificity was checked using melting curve analysis following the manufacturer's instructions. Data analysis utilized the GeneAmp 5700® SDS Software (version 1.3) to determinate the threshold cycle (C_t) for each amplified product.

Cell extracts and high-performance liquid chromatography (HPLC) - Each purified parasite stage was freeze-dried and successively extracted with hexane (3 × 0.5 ml) and ethanol/water (1:1, v/v; 1 × 1 ml at 55ºC for 1.5 h). Aliquots of each extract were monitored for radioactivity with a Beckman LS 5000 TD β-counter. Metabolic analyses by HPLC of DOXP, pyridoxal 5'-phosphate (PNP), MEP, CDP-ME, CDP-MEP, MEcPP, dolichols and ubiquinones were carried out as described previously (Cassera et al. 2004).

RESULTS

Metabolic and transcript profiles of the MEP pathway during the intraerythrocytic cycle - The metabolite and transcript levels of the intermediates of the MEP pathway were analyzed to investigate the relationship between the intermediate levels and the metabolic requirement for the isoprenoid biosynthesis (dolichols and ubiquinones) throughout the intraerythrocytic cycle of *P. falciparum* (Fig. 2). Since GAP is also direct precursor in pyridoxal 5'-phosphate (PNP) biosynthesis (Fig. 1), PNP was included in our analyses (Burns et al. 2005, Gengenbacher et al. 2006, Zein et al. 2006). The metabolite levels of all analyzed intermediates showed an

TABLE I

Gene-specific oligonucleotides used for the real-time polymerase chain reaction transcript analysis

Gene	PlasmoDB ID	Primers	Amplicon coordinates	Length (base pairs)
DXS	MAL13P1.186	F: 5´-CTTTAAAGGTTATAATTGGAAGAAGTGGA-3´ R: 5´-GTGTTGCCCCATCCTCTCC-3´	2768 - 2821	3618
DXR	PF14_0641	F: 5´-AAAACCTTTAGATTTGGCTCAGGTT-3´ R: 5´-GTTGTTAGCTATTTCATTTGACGCA-3´	1134 - 1284	1467
MCT	PFA0340w	F: 5´-ACTGAATTGATCGGTCCTAAGCA-3´ R: 5´-TTATACTGTGGATGATACATGAAAAATGTT-3´	625 - 775	2205
CMK	PFE0150c	F: 5´-TGGCTCATCTAATGGTGCTACTG-3´ R: 5´-TCCGTACAATAAGCAAATCCAGAAC-3´	876 - 1028	1614
MCS	PFB0420w	F: 5´-TTTTAGGTGCCTTAGGTTCTTTAGACAT-3´ R: 5´-ACATTAATATCCACGTTCCCAATATCA-3´	386 - 536	723
GcpE	PF10_0221	F: 5´-TGCTGACATTGTAAGGTTGACTGTT-3´ R: 5´-ACACATCAGCTGCCATTAAAGC-3´	492 - 643	2475
LytB	PFA0225w	F: 5´-AATCAGAAATGTTCCAGCAGTATTACTT-3´ R: 5´-TGGAGGGTTTGTTAATAGGTTGACA-3´	1356 - 1506	1608
FPPs	PF11_0295	F: 5´-TGAGTGGGAAAAAGTGGCTTGTA-3´ R: 5´-CACCGCATTCTTAATTTCAACGT-3´	192 - 342	1110
PDX1	MAL6P1.215	F: 5´-TTGTTAATTTTGCTGCTGGAGGT-3´ R: 5´-TTGCTAACAGCTGAAACGATTGA-3´	626 - 776	906
Lsa 1	PF10_0356	F: 5´-CAGAAAATGAACGTGGATATTATATACCA-3´ R: 5´-TGTATATCCCTTCGTCCTTCAACAT-3´	4055 - 4205	4791

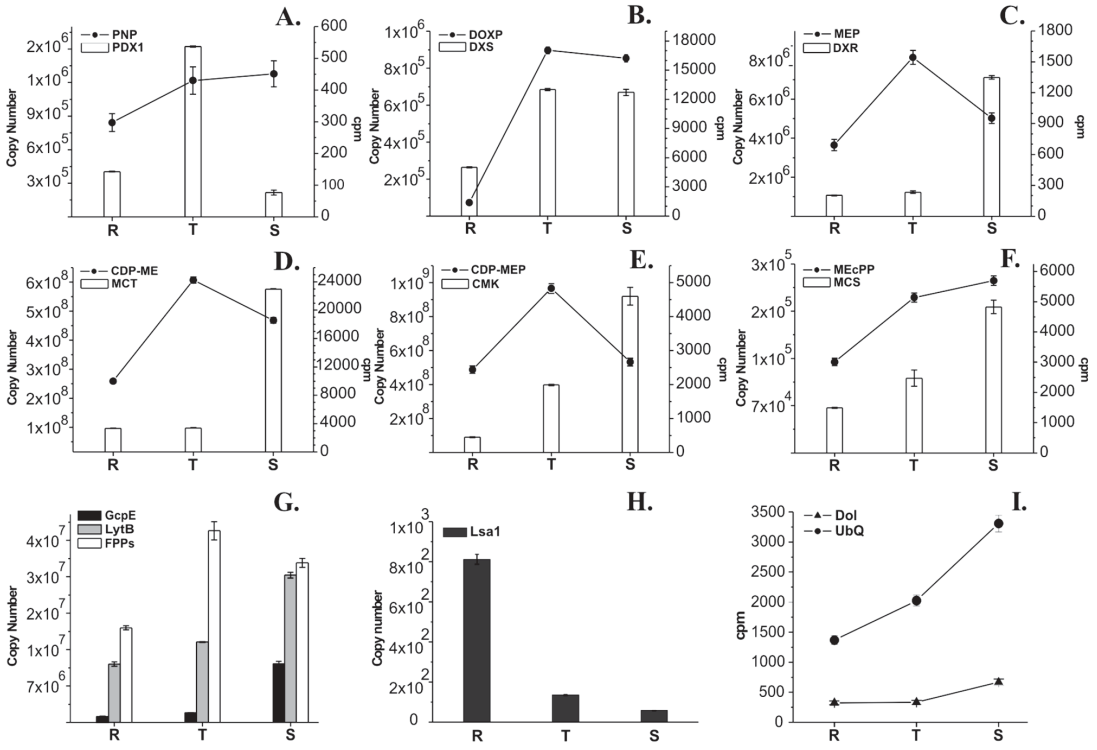

Fig. 2: metabolite and transcript profiles of each analyzed intermediate and its metabolizing enzyme during the intraerythrocytic cycle of *Plasmodium falciparum*. Panels A to F show the metabolite profile (line) and transcript level (bar) for the first five intermediates of the MEP pathway and PNP. Panels G and H display the transcript profiles for the GcpE, LytB, FPPs, and Lsa-1 genes. Dolichol and ubiquinone metabolite profiles are shown in panel I. Ring (R), trophozoite (T) and schizont (S). The absolute transcript quantities per 100 ng converted RNA are indicated. Metabolite quantities are given in counts per minute (cpm).

increase from the ring to the trophozoite stage (Fig. 2A-F, line). In the schizont stage, levels of PNP, DOXP, and MEcPP were maintained (Fig. 2A, B and F, line), but the levels of MEP, CDP-ME and CDP-MEP decreased relative to the trophozoite stage (Fig. 2C-E, line). Dolichol levels showed only a slight increase towards the schizont stage but the ubiquinone level was significantly increased from the ring to the schizont stage (Fig. 2I).

The intermediates HMBPP, IPP, DMAPP and geranyl pyrophosphate (GPP) were not resolved in the HPLC system used in our analyses. Nevertheless, the corresponding genes *GcpE*, *LytB*, and *FPPs* were included in the transcript analysis (Fig. 2G). Quantification and transcript analysis of these and the MEP pathway genes revealed different transcript profiles. Interestingly, except *DXS*, all genes involved in the MEP pathway (*DXR*, *MCT*, *CMK*, *MCS*, *GcpE*, and *LytB*) had peak transcript abundance in the schizont stage (Fig. 2B-G, bar). *DXS* transcripts showed an increase in trophozoites and were maintained in schizont stage (Fig. 2B, bar). *PDX1* and *FPPs* transcript profiles displayed a maximum in trophozoite (Fig. 2A and G bars). The *Lsa-1* gene is normally not expressed in the erythrocytic cycle and was employed as a negative control. *Lsa-1* transcripts were almost undetectable in trophozoite and schizont stages and the quantity detected in ring stage was very low (Fig. 2H), possibly due to relaxed transcription in ring stage (Blair et al. 2002).

Effect of fosmidomycin on the metabolic and transcript profiles - To evaluate if changes in the metabolic activity of the MEP pathway affect the transcript levels (Table II), we investigated the effect of fosmidomycin on metabolite and transcript levels. In general, the effect of fosmidomycin on the biosynthesis of the MEP pathway intermediates (DOXP to MEcPP), dolichols and ubiquinones was mainly observed in ring stages, where DOXP was accumulated, whereas MEP and downstream intermediates were observed in decreased levels. The most substantial effect on the end products of the isoprenoid biosynthesis was observed on the ubiquinones. Similar results, but less evident, were obtained in the schizont stage. The effect on the steady-state RNA levels of the analyzed genes was greater in the schizont stage, where the transcripts were accumulated, except *MCT*. A slight effect of fosmidomycin in the trophozoite stage was observed on metabolite and transcript levels. Interestingly, we observed that transcript level of *LytB*, which codes for the last enzyme of the MEP pathway, was not altered. *FPPs* transcript levels were only affected in the schizont stage, where 30% increase of transcripts was detected in fosmidomycin-treated parasites. The level of the *PDX1* transcripts but not of the PNP metabolite was affected by fosmidomycin treatment in ring-stage parasites. In trophozoite and schizont stages both PNP metabolite and *PDX1* transcript levels were increased.

TABLE II

Effect of 1 μM of fosmidomycin on the metabolite and transcript levels of each analyzed intermediate and its respective metabolizing enzyme at the three characteristic developmental stages of *Plasmodium falciparum*

	Ring (% effect)		Trophozoite (% effect)		Schizont (% effect)		
Intermediate	Metabolite	Transcript	Metabolite	Transcript	Metabolite	Transcript	Gene
PNP	0	-45	16	5	28	30	*PDX1*
DOXP	4	-19	-2	0	-23	50	*DXS*
MEP	-13	0	0	-17	-15	16	*DXR*
CDP-ME	-35	33	-2	10	-26	-5	*MCT*
CDP-MEP	-30	35	0	-5	-7	33	*CMK*
MEcPP	-41	0	-35	19	0	34	*MCS*
HMBPP	–	39	–	-3	–	31	*GcpE*
IPP / DMAPP	–	-7	–	0	–	0	*LytB*
GPP	–	0	–	0	–	30	*FPPs*
Ubiquinones	-24	–	-11	–	-18	–	–
Dolichols	-2	–	0	–	0	–	–

Positive values indicate increase of the intermediate or transcript levels and negative values indicate reduction of the intermediate or transcript levels compared to corresponding untreated control samples. A zero indicates no-effect. (–): not analyzed.

DISCUSSION

This is the first analysis that compares the effect of fosmidomycin on the metabolic and transcript profiles in *P. falciparum*, which has only the MEP pathway for IPP and DMAPP biosynthesis. A combined analysis of metabolic and transcript profiles may be a useful procedure for the identification of candidate enzymes as new drug targets (Urbanczyk-Wochniak et al. 2003).

Following the discovery of the MEP pathway, several studies have been conducted to understand the regulation of this metabolic route. Fruit development in the tomato has been widely used as a system for these studies due to the observation that a significant increase in the supply of isoprenoid precursors is required to permit the massive accumulation of carotenoids that takes place during ripening. In this process, MEP-pathway key enzyme transcripts are accumulated; meanwhile, the transcript levels of the other MEP enzymes remain unchanged (Lois et al. 2000, Rodriguez-Concepcion et al. 2001, 2003, Botella-Pavia et al. 2004). Despite the differences, *P. falciparum* intraerythrocytic development may be compared with tomato fruit ripening, since malaria parasites showed an increased biosynthesis of isoprenoids towards the end of schizogony (Fig. 2I). Therefore, using an approach similar to employed monitoring carotenoid synthesis during the fruit development in the tomato, we chose ubiquinones as the biological marker of isoprenoid precursor requirement from the MEP pathway in *P. falciparum*. Importantly, the comparative study of metabolite biosynthesis was conducted using the same absolute number of parasites, obtained from highly synchronized stages. In fact, ubiquinone levels were increased at the end of the intraerythrocytic cycle when the parasite is preparing for schizogony. Dolichol, the other isoprenoid studied herein, did not show significant variation in its levels during the intraerythrocytic cycle of the parasite, and its overall quantity detected was very low (Fig. 2I). This was expected because the main function of dolichols, as dolichyl phosphate, is their participation in glycoprotein synthesis in the endoplasmic reticulum, where the dolichol carrier is recycled (Chojnacki & Dallner 1988, Burda & Aebi 1999).

In this framework, the metabolite levels of most of the intermediates of the MEP pathway showed an increase in trophozoite and a decrease in schizont stages, when ubiquinones were accumulated. Interestingly, metabolic profiles showed that DOXP and CDP-ME were highly accumulated when compared to the other intermediates, mainly in the trophozoite and schizont stages (Fig. 2).

We considered that both DOXP and CDP-ME could act as a metabolite reserve, which might be used in the schizogony to sustain the high demand of isoprenoids, and both intermediates might be key metabolites of the MEP pathway in *P. falciparum*.

Metabolic results were correlated with the transcript profiles of genes involved in the MEP pathway. In general, transcript profiles of MEP pathway genes as well as *PDX1* and *FPPs* were similar to the results obtained by *P. falciparum* microarray studies (Le Roch et al. 2003, Llinas et al. 2006), taking in to consideration that the resolution of quantitative real time PCR is higher than the microarray technique. All genes involved in the MEP pathway had maximal transcript quantities in the schizont stage. Of those, *MCT* and *CMK* transcripts were always the most abundant in all stages (Fig. 2). Maximal transcript levels in the schizont stage were concomitant with an increase in the ubiquinone biosynthesis and a reduction of the respective MEP pathway intermediate. Several studies showed that in tomato fruit ripening the accumulation of carotenoids only requires increased levels of *DXS*, *DXR*, and *LytB* transcripts (Lois et al. 2000, Rodriguez-Concepcion et al. 2001, 2003, Botella-Pavia et al. 2004). Interestingly, we observed a positive correlation in *P. falciparum* between enhanced isoprenoid biosynthesis (ubiquinones) and accumulation of transcripts encoding all enzymes of the MEP pathway except for *DXS*.

The effect of fosmidomycin treatment was stage-dependent both at RNA transcripts and metabolite levels. Treatment with 1 μM of fosmidomycin resulted, mainly in the ring stage, in a decrease in the intermediate biosynthesis of the MEP pathway as well as in the ubiquinone and dolichol biosynthesis as compared to controls. Slight differences were observed using asynchronous cultures in our previous work, predominantly in schizont stages (Cassera et al. 2004). In fosmidomycin-treated parasites, an increase of the transcript levels of the MEP pathway genes was mainly observed in the schizont stage. The general effect of fosmidomycin on the transcript levels of the analyzed genes was small (Table II). This effect is very similar to what was observed in previous studies when parasites were treated with pyrimethamine (Altschul et al. 1997, Gunasekera et al. 2003). These findings and our results reinforce the point of view that drug-target finding in *P. falciparum* is very difficult using only transcript-based analysis.

We observed a reduction of the level of *PDX1* transcripts by fosmidomycin treatment in ring-stage parasites but the PNP metabolite was not affected; while in trophozoite and schizont stages both PNP metabolite and *PDX1* transcript levels were increased. The substrates for *P. falciparum* PDX1 (ribose 5-phosphate and ribulose 5-phosphate and GAP as well as dihydroxyacetone) and its cytosolic localization have been recently characterized (Gengenbacher et al. 2006). The intermediate GAP is also substrate of DOXP synthase (Fig. 1). In *P. falciparum*, the MEP pathway occurs in the apicoplast, where the substrates for the DOXP synthesis are imported from the cytosol as phosphoenolpyruvate and dihydroxyacetone phosphate (DHAP). The DHAP is converted into GAP in a reversible reaction and can be transport outside of the apicoplast (Ralph et al. 2004 and references herein). Therefore, changes on the MEP pathway metabolites could affect the cytoplasmatic GAP and PNP levels.

Since the main effect of fosmidomycin treatment on the metabolic and transcript profiles was observed at different times during the developmental cycle and the effect on the transcript levels, we suggest a post-transcriptional regulation of the MEP pathway in *P. falciparum*.

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