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Effect of tamoxifen on the sphingolipid biosynthetic pathway in the different intraerythrocytic stages of the apicomplexa *Plasmodium falciparum*

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ABSTRACT

Parasites of the genus *Plasmodium* responsible for Malaria are obligate intracellular pathogens residing in mammalian red blood cells, hepatocytes, or mosquito midgut epithelial cells. Regarding that detailed knowledge on the sphingolipid biosynthetic pathway of the apicomplexan protozoan parasites is scarce, different stages of *Plasmodium falciparum* were treated with tamoxifen in order to evaluate the effects of this drug on the glycosphingolipid biosynthesis. Thin layer chromatography, High performance reverse phase chromatography and UV-MALDI-TOF mass spectrometry were the tools used for the analysis. In the ring forms, the increase of NBD-phosphatidyl inositol biosynthesis was notorious but differences at NBD-GlcCer levels were undetectable. In trophozoite forms, an abrupt decrease of NBD-acylated GlcDHCer and NBD-GlcDHCer in addition to an increase of NBD-PC biosynthesis was observed. On the contrary, in schizonts, tamoxifen seems not to be producing substantial changes in lipid biosynthesis. Our findings indicate that in this parasite, tamoxifen is exerting an inhibitory action on Glucosylceramidesynthase and sphingomyelin synthase levels. Moreover, regarding that *Plasmodium* does not biosynthesize inositolphosphoceramides, the accumulation of phosphatidylinositol should indicate an inhibitory action on glycosylinositol phospholipid synthesis.

1. Introduction

Malaria is a major healthcare problem worldwide that remains an important cause of morbidity and mortality. Approximately 214 million malaria cases and 438 000 malaria-related deaths were reported globally in 2015 [1]. Nowadays, malaria control is entirely dependent on pharmacological treatments. Thus, an unceasing necessity to identify new drug targets involved in relevant parasite metabolic pathways takes place [2].

Sphingolipids (SLs) are amphipathic lipids comprising sphingosine as the building unit *N*-acylated with a long-chain fatty acid (*i.e.* ceramide) and substituted with a head group moiety (sphingomyelin (SM); glucosylceramide; ceramide-1-phosphate) [3]. They are a complex class of signaling molecules and an essential part of cellular membranes. Their levels regulate proliferation, apoptosis, and inflammation depending on the specific sphingolipid species, cell and receptor type, and intracellular targets. They are essential for specific membrane functions [4] and directly modulate intracellular specific effector proteins [5–7]. Ceramides, tightly regulated in the cells, are generated in different cellular compartments by three different pathways: the *de novo* pathway in the endoplasmic reticulum, the salvage/SMase pathway in the Golgi, lysosome, and the plasma membrane, as well as by recycling of GSLs. There is some evidence that alterations in SL metabolism, leading to enhanced ceramide production, occur in neurological disorders [8–10]. Cytokines such as tumor necrosis factor-alpha [11] and reactive oxygen species [12] induce the production of ceramide through activation of SMases. Therefore, the activation of

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the SMase pathway is believed to be a general cellular stress response.

Mammals produce SM as the primary complex sphingolipid. In contrast, yeast, plants and some protozoa use an evolutionarily related inositol phosphorylceramide (IPC) synthase to synthesize IPC. This activity has no equivalent in mammals and IPC synthase has been proposed as a target for anti-protozoans.

In the apicomplexa *P. falciparum* and *T. gondii*, it has been demonstrated that SLs are synthesized *de novo* [13,14]. As in mammals, *P. falciparum* synthesize SM [15–17]. In *T. gondii* the synthesis of SM has been also observed but at a lower proportion in comparison with the level of glycosphingolipids (GSLs) synthesis [14–18]. The presence of an active glucosyl ceramide synthase (GCS) in the intraerythrocytic stages of *P. falciparum* has been described [19]. Interestingly the parasite was capable to glycosylate only dihydroceramide. In accordance, among sphingolipid subclasses *in P. falciparum* presence of dehydroSM (dhSM) and dihydroCer (dhCer) was recently documented [20]. In addition to the *de novo* synthesis, the recycling or salvage *via* of SLs from their precursors in the host cell have been also observed in *Apicomplexa* [21].

Many SLs play important roles in processes related with the programmed cell death (PCD) mechanisms as well as in cellular survival. Chemotherapeutic and radiation treatment, increase ceramide levels inducing apoptosis. In certain tumor types, such as mammal breast, ceramide levels attenuation by action of the GCS generates glucosylceramide from ceramide, triggering drug resistance. The ceramide formed in the plasmatic membrane may occur in the lipid rafts. This ceramide may affect signaling *vias* generated by receptors grouped in rafts such as fatty acid synthase [22]. By contrast, the sphingosine-1-phosphate (S-1-P) is an anti-apoptotic molecule that mediates in a great amount, antagonic cellular effects to the others pro-apoptotic SLs. These lipidic mediators are metabolically juxtaposed, suggesting that their metabolic regulation is very important in determining the cellular destination [23]. Taking into account that sphingosine 1-phosphate bases are mitogenic and anti-apoptotic [24] they might act as protective against apoptosis due to the *de novo* synthesis of ceramide [25]. However, it is clear that the *de novo* synthesis of SLs participates in the induced cellular death by a wide variety of agents [26,27]. In the last two decades, the CerSs have been implicated in PCD control. Disruption of an important metabolic pathway in the parasite can incite it to undergo apoptosis-like cell death [28]. Different anti-malarial drugs, antibiotics and other small molecules can develop the induction of apoptosis-like cell death in *P. falciparum* [29,30].

In this line, the aim of this work was to evaluate the effects of tamoxifen in the different intraerythrocytic stages of *Plasmodium falciparum* in order to determine the effect of this drug on glycosphingolipid biosynthesis. Thin layer chromatography, high performance reverse phase chromatography and UV-MALDI-TOF mass spectrometry were the tools used for the analysis. Our findings indicate that in this parasite, tamoxifen should be exerting an inhibitory action on GCS and sphingomyelin synthase levels although in a stage dependent way.

2. Materials and methods

2.1. Materials

NBD-ceramide (NBD-Cer) and NBD-DHceramide, lipid standards and BSA were purchased from Sigma. AlbuMax I® was purchased from Gibco BRL Life Technologies (NY, USA) and Percoll® Pharmacia Chemicals (Uppsala, Sweden). TLC was performed on silica gel 60 pre-coated plates (Merck) using solvent systems: (a) chloroform/methanol/water (40:10:1, v/v/v) and (b) propanol/NH_3/H_2O (75:5:5, v/v/v).

2.2. Parasite culture

An isolate NF54, clone 3D7 of *P. falciparum* was used. Parasites were cultured according to Trager and Jensen with modifications [31,32]. The gas mixture of the tissue culture flasks (75 cm^2) contained 5.05% CO_2, 4.93% O_2 and 90.2% N_2.

Starting with asynchronous cultures, parasites were cultured in fresh red blood cells depleted of leukocytes by treatment with 6% (wt/vol) Plasmagel® (Laboratoire Roger Bellon, Neuilly sur Seine, France) in physiological saline [33]. They were washed and resuspended in RPMI 1640 medium containing 0,5% albumax®. Ring (1–20 h after reinvasion), trophozoite (20–30 h after reinvasion) and schizont (30–45 h after reinvasion) forms were purified on a 40/70/80% discontinuous Percoll® (Pharmacia LKB Uppsala Sweden) density gradient [32]. Parasite development, multiplication and red blood cells were monitored by microscopic evaluation of Giemsa stained thin smears.

2.3. Treatment of parasites with tamoxifen

Parasite cultures were incubated with 10 μM of tamoxifen citrate [34]. After 24 h of treatment, parasites (6% ring stages, 4% trophozoite stages, 4% schizont stages) were labelled with NBD-ceramide or NBD-DHceramide previously coupled to BSA at a concentration of 5 μM in RPMI 1640 medium for 24 h.

2.4. Isolation and purification of glycosphingolipids

After lyophilization, each labelled intraerythrocytic stage was extracted with chloroform/methanol 1:1 (3 × 1 ml). Each extract was fractionated by anionic exchange chromatography on DEAE-Sephadex A-25 (acetate form) column [19].

2.5. High performance liquid chromatography analysis

The analysis was performed in a Waters 600 liquid chromatograph equipped with a multi fluorescent detector Waters 2475. A reverse phase RP-18 (Supelco, 5 μm) column with methanol: water (9:1, v/v) as mobile phase was used at a 0.5 ml/min flow, λexc = 465 nm and λem = 530 nm.

2.6. MALDI-TOF mass spectrometry analysis

Mass spectrometry analysis was performed in an Ultraflex II TOF/TOF mass spectrometer equipped with a High performance solid state laser (λ = 355 nm) and a reflector, operated by the Flexcontrol 2.4 software package (Bruker Daltonics, GmbsH, Bremen, Germany). Samples were irradiated with a laser power of 40%, measured in the linear and the reflectron modes, in positive and negative polarity. The samples were loaded onto a ground steel sample plate (MTP 384 ground steel; Bruker Daltonics GmbsH). Mass spectra were the sum of 100–500 single laser shots, depending on the sample conditions. External calibration: commercial proteins bradykinin 1–7, MW 757.399; angiotensin I, MW 1296.685; renin substrate, MW 1758.933; and insulin β-chain, MW 3494.6506 with CHCA as matrix in positive and negative ion mode were used.

3. Results

3.1. Inhibition assays with tamoxifen in Plasmodium falciparum cultures

Cultures containing 5% hematocrit and 5% parasitaemia were grown in the presence and absence of tamoxifen (TAM, 10 μM). A

non-parasitized erythrocyte culture was used as negative control. After 24 h of incubation NBD-DH-Cer or NBD-Cer 5 μM was added to the culture medium and was incubated 24 h more.

3.2. Lipid analysis by thin layer chromatography (TLC)

Lipid extraction and fractionation was performed and the fraction of neutral and zwitterionic lipids was analyzed using chloroform: methanol: water (40:10:1 v/v/v) as development solvent. In all cases, analysis of lipids obtained from equal number of parasites treated and untreated was evaluated. Fig. 1 shows the neutral lipidic pattern of *P. falciparum* schizont stage metabolically incorporated with each precursor with or without TAM treatment. Interestingly, the effect of TAM shown with each fluorescent precursor was different: while using NBD-DH-Cer as precursor, SL biosynthesis increased, with NBD-Cer, SL biosynthesis resulted notably diminished after treatment. However, in the last case, all the fluorescent precursor was metabolized as no remnant NBD-Cer is observed. Considering that the drug does not affect the fluorescent precursor incorporation, this fact would indicate that NBD-Cer is not used for the biosynthesis of complex SL, but it is hydrolyzed to sphingosine to achieve further on, complete degradation. It must be noted that the loss of the fluorescent group precludes the detection of this transformation. On the contrary, it was also shown that the NBD-DHCer used as precursor was in excess although the lipid metabolism increased by TAM treatment.

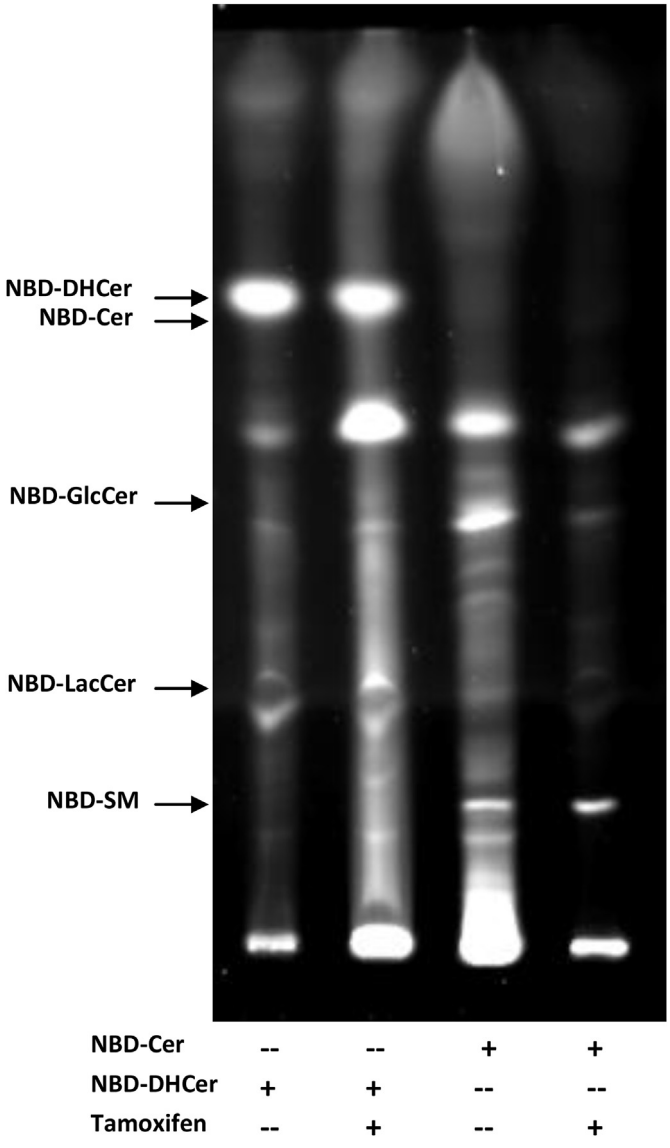


Fig. 1. Neutral lipid analysis of schizont forms of *P. falciparum* in the presence or absence of tamoxifen. TLC was developed in solvent system a. In all cases 8×10^7 parasites were analyzed.

Further on, the action of TAM in the different intraerythrocytic stages of the parasite was investigated. In this case, the use of the fluorescent dihydroceramide was considered taking into account that it has been previously demonstrated that GCS acts on this precursor in *P. falciparum* [19]. TLC analysis of neutral lipids extracted from control and TAM treated-parasites are shown (Fig. 2A). Notable differences in the profile among the different stages and between non-treated and treated parasites were found. First, in all stages, NBD-dihydroceramide used as precursor of the biosynthesis of sphingolipids was maintained in excess despite the lipids suffered changes with marked differences depending of each particular stage, indicating that tamoxifen exerted a differential action on each of them. It was interesting to note that, rings treated with tamoxifen presented an increase in the biosynthesis of neutral lipids in comparison with control rings (Fig. 2A, lanes 2 and 5). Band F presenting higher mobility than that corresponding to the NBD-GlcDHCer, slightly decreased in treated rings, but an increase in the biosynthesis of bands named G and H was observed.

Regarding the trophozoite fraction, TAM exerted a marked inhibitor action on sphingolipids biosynthesis, being this stage one of the most actives in normal culture conditions. The disappearance of band F, in addition to a minor biosynthesis of the band corresponding to NBD-DHCer and a slight increase of the band H with Rf similar to NBD-SM, was observed in TAM-treated parasites (Fig. 2, lanes 3 and 6). Treated schizont forms evidenced a slight increase of the synthesis of the G and H bands with respect to the control. (Fig. 2A, lanes 4 and 7).

The analysis of acidic lipids by TLC using solvent system b, is shown in Fig. 2B. Only the trophozoite stage is shown taking into account that sulfoglycosphingolipids are predominant in the trophozoite stage as previously described [35]. The disappearance of the band corresponding to the SPf1 in treated trophozoites was notable and would be in accordance with the fact that neutral lipids are diminished.

3.3. High performance reverse phase chromatography analysis

Samples purified from the different intraerythrocytic stages were analyzed by HPLC using a reverse phase C18 column and methanol: water, 9:1, v/v as mobile phase. In Fig. 3 the chromatograms corresponding to the labelled sphingolipid samples from TAM-treated and control parasites are shown. It is worth noting that in order to detect the components present in each fraction, and collect them individually for further mass spectrometry analysis; the optimization of the samples dilutions was first developed. In ring stage (Fig. 3a, b), in the TAM-treated sample, the presence of a compound at Rt = 5.82 min was evidenced which had not appeared in the same stage without treatment. Control trophozoites (Fig. 3c, d) showed a high level of biosynthesis in accordance with the observed by TLC. In this case, TAM diminished the biosynthesis of compounds with lower retention times, increasing the proportion of NBD-DHCer. The clear definition of the chromatographic peaks favored the collection of each of them for further analysis by mass spectrometry. In the schizont stage (Fig. 3e, f), treatment with TAM showed the presence of little amounts of NBD-GlcDHCer (Rt = 9.86), PC (Rt = 16.20) and of a component of Rt = 5.46 that was not observed in the sample without treatment.

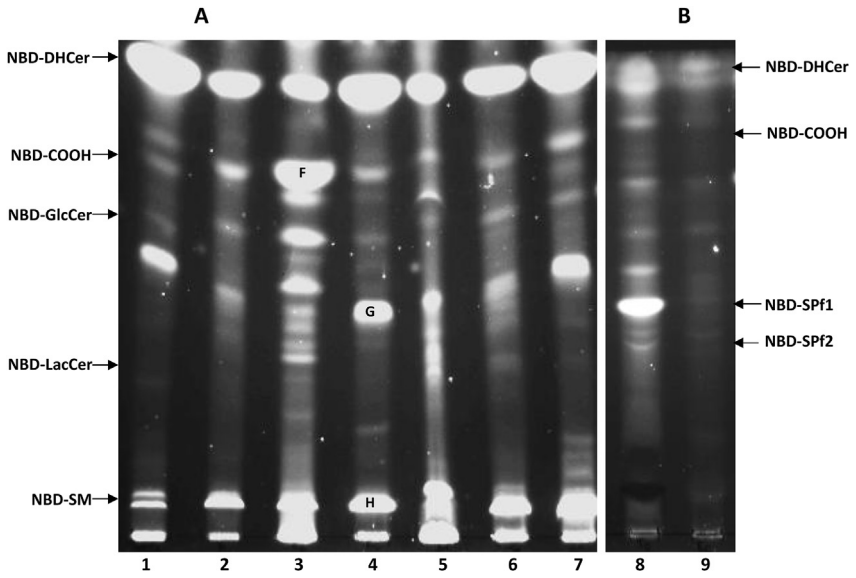


Fig. 2. TLC analysis of lipids extracted from the different intraerytrocytic stages of *P. falciparum* treated or not with tamoxifen. A) neutral lipids (solvent system a), B) acidic lipids (solvent system b). Lane 1-non infected erythrocytes; lanes 2, 3 and 4 untreated rings, trophozoites and schizonts respectively; lanes 5, 6 and 7, tamoxifen treated rings, trophozoites and schizonts respectively. Lanes 8 and 9 control and treated trophozoites respectively. Results shown are representative of three different experiments.

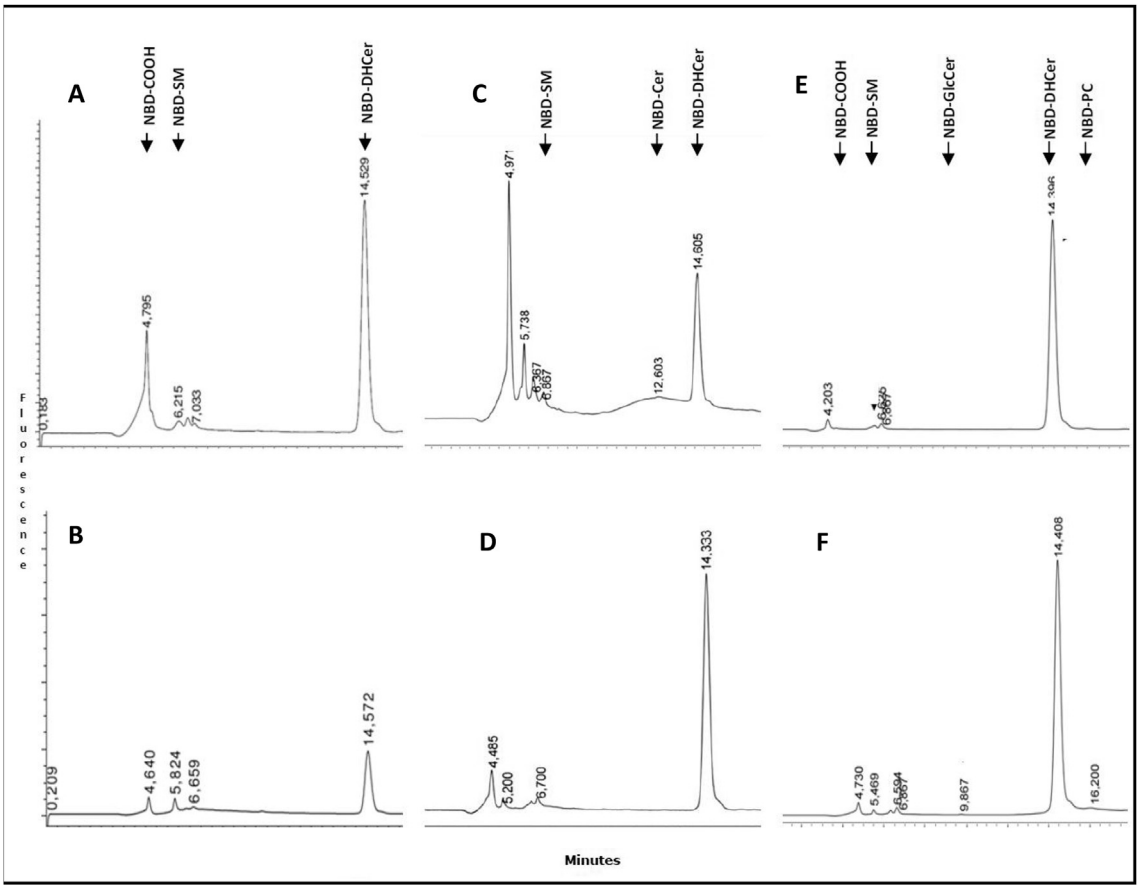


Fig. 3. HPLC analysis of lipids extracted from the different intraerytrocytic stages of *P. falciparum*. A, C and E–control ring, trophozoite and schizont forms, respectively. B, D and F-tamoxifen treated ring, trophozoite and schizont forms, respectively.

3.4. MALDI-TOF mass spectrometry analysis

The identity of the products obtained in the different stages was confirmed by UV-MALDI-TOF mass spectrometry. As some examples, Fig. 4A corresponds to the spectra of band F (from Fig. 2) obtained from control trophozoites performed using GA as matrix.

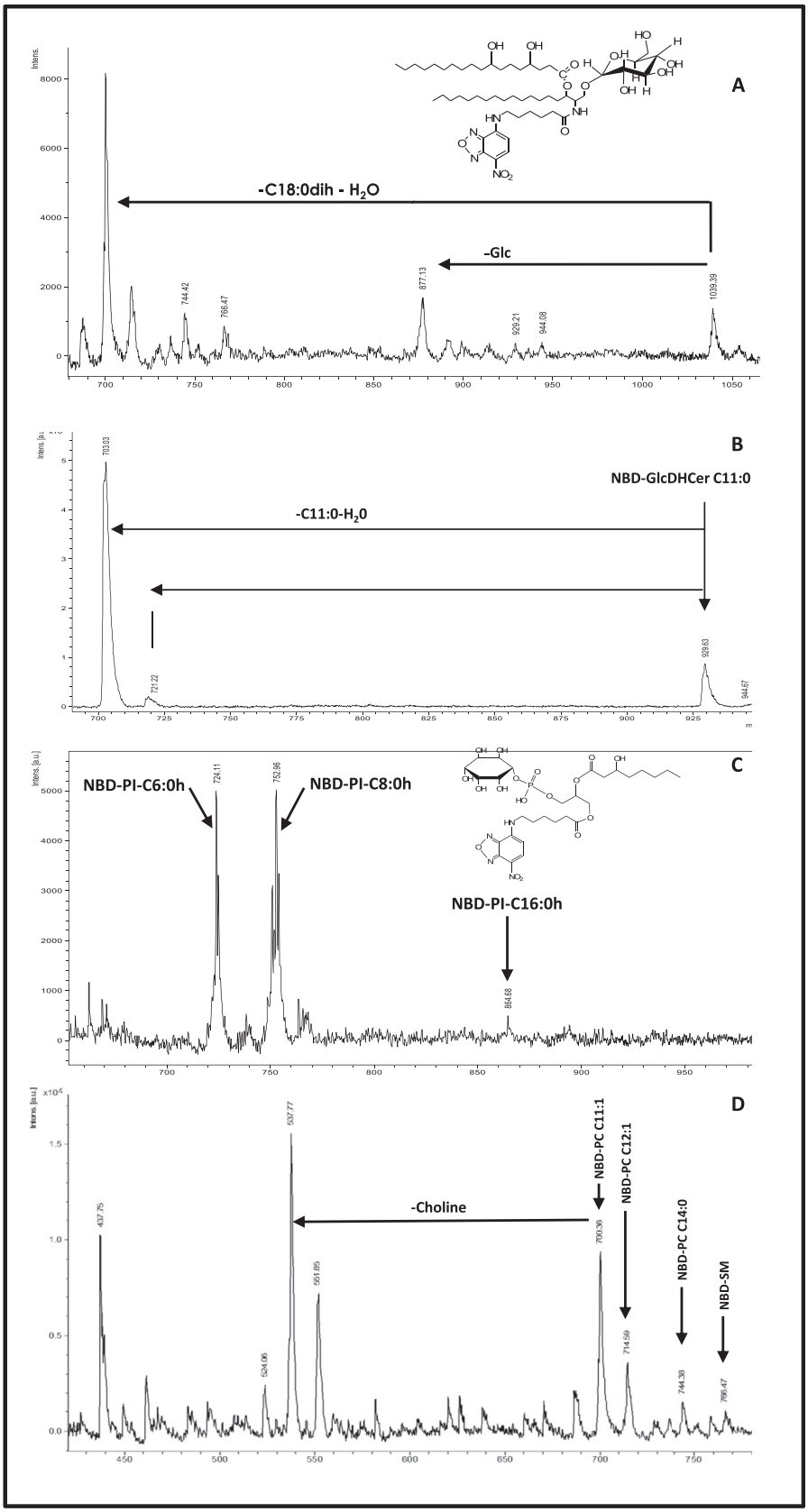


Fig. 4. MALDI-TOF mass spectra of isolated lipids from Fig. 2 and 3. A-spot F from Fig. 2A; obtained from control trophozoites performed using GA as matrix. B- peak Rt = 4.97 min from Fig. 3A; C- Spot G from Fig. 2A from control schizonts; D- Spot H from Fig. 2A from control schizonts.

Signals corresponding to acylated NBD-GlcDHCer with different fatty acids were observed. The ion *m/z* 1039.39 (calc. *m/z* 1039.36) was attributed to NBD-GlcDHCer acylated with C18:0dih fatty acid. The signal *m/z* 877.12 (calc. *m/z* 877.22) corresponds to the loss of a glucose and *m/z* 703.03 (calc. *m/z* 704.43) corresponds to the loss of a fatty acid and a water molecule from ion *m/z* 1039.39.

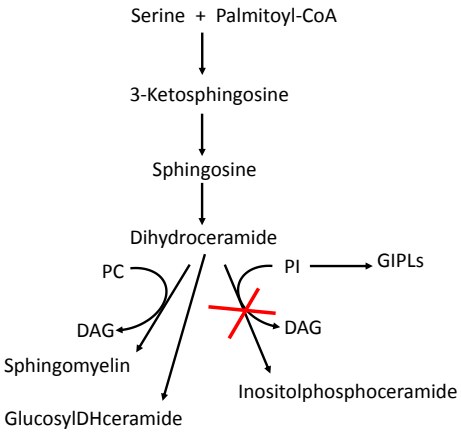
Fig. 4B shows the analysis of peak with Rt = 4.97 from the HPLC analysis from Fig. 3a. The presence of an acylated NBD-GlcDHCer was observed in coincidence with the results presented above. Thus, ion *m/z* 929.63 (calc. *m/z* 930.58) corresponds to NBD-GlcDHCer acylated with C11:0 and *m/z* 703.03 (calc. *m/z* 704.43) is ascribed to the loss of the fatty acid and a molecule of water. The minor signal at *m/z* 944.66 (calc. *m/z* 945.19) may be attributed to a C12:0-fatty acid acylated-NBD GlcDHCer. The differences in the fatty acid acylating glucosylceramide might be due to the use of an HPLC isolated peak for the analysis. Fig. 4C shows the MALDI-TOF m.s. spectrum obtained from band G (Fig. 2) using CHCA as matrix. Signals corresponding to ions *m/z* 724.10 (calc. *m/z* 724.06), *m/z* 752.96 (calc. *m/z* 751.27) and *m/z* 864.68 (calc. *m/z* 863.40) were attributed to NBD-phosphatidylinositol containing the fatty acids C6:0h, C8:0h, and C16:0h respectively. In treated trophozoites, MALDI-TOF analysis of spot H, (Fig. 2), displayed signals at *m/z* 744.35 (calc. *m/z* 744.39) attributed to NBD-PC-containing a C14:0 fatty acid; *m/z* 714.58 (calc. *m/z* 714.35) attributed to the NBD-PC-containing a C12:1 fatty acid and *m/z* 700.35 (calc. *m/z* 700.33) ascribed to the same compound bearing a C11:1 fatty acid. Ion at *m/z* 536.77 (calc. *m/z* 535.28) corresponding to the loss of a phosphocholine group from the ion *m/z* 700.35 and *m/z* 437.42 (calc. *m/z* 437.25) to the loss of the NBD group from *m/z* 714.58, confirmed the mentioned structures. The presence of NBD-DHSM was also determined as a signal at *m/z* 766.47 (calc. *m/z* 765.43) was detected.

4. Discussion

The viability of *P. falciparum* intraerythrocytic stages is dependent of lipid synthesis, uptake, and transport [36]. In addition, *P. falciparum* suffers along its life cycle a rapid proliferation driven by *de novo* synthesis and acquisition of host cell lipids. As sphingolipid metabolism is a dynamic process with complex orchestration, impact, and clinical applications, it presents druggable targets for exploitation. The *Plasmodium* sphingolipid metabolic pathway is known to have activity of several enzymes [19,37–39].

Tamoxifen, has been described as an effective inhibitor of sphingolipid metabolism in mammalian systems, therefore it is an interesting agent for studying the sphingolipid biosynthetic pathway in parasites. Although it has been determined that TAM has no effect on parasite growth [40], in this work, the treatment of the three different intraerythrocytic stages of *P. falciparum* with TAM has demonstrated that the drug triggers interesting differential effects in the bioactive sphingolipid pathway.

In the ring forms, the increase of NBD-phosphatidyl-acylated inositol biosynthesis was notorious but differences at glucosylceramide levels were undetectable. Regarding that *Plasmodium* does not biosynthesize IPC [14], the accumulation of PI would indicate an inhibitory action on GPI synthesis (Scheme 1). In *Apicomplexa*, precursor GPI lipids are believed to play important roles as toxins and immune modulators [41,42]. In trophozoite forms, an abrupt decrease of NBD-acylated GlcDHCer and NBD-GlcDHCer in addition to an increase of NBD-PC was observed. These results indicate that TAM would be exerting an inhibitory action on GCS and SM synthase. In this sense, when PPMP was added to parasite cultures, a correlation between arrest of parasite growth and inhibition of glycosphingolipid biosynthesis was observed [19]. Recent studies on phosphoinositides have highlighted important functions of



Scheme 1. Sphingolipid biosynthetic pathway in *P. falciparum.*

phosphoinositide kinases in several parts of the *Plasmodium* life cycle such as cytokinesis during the erythrocytic stage [43].

On the other hand, in schizonts, TAM seems not to be producing notorious changes in lipid synthesis although PC is also slightly increased. In addition, the fact that TAM exerts a differential effect on sphingolipids incorporated with NBD-DHceramide and NBD-Ceramide evidence that these two metabolites are not interconverting.

In conclusion, the comparative analysis of *P. falciparum* sphingolipid metabolism performed in the different parasite asexual forms highlighted a differential sphingolipid pathway in each stage offering important insights into parasite survival strategies that may open new ways for expanding their potential use as therapeutic targets.

Conflicts of interest

The authors declare that this manuscript has no conflict of interest.

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