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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

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