

**Cytotoxicity of myriocin against axenic culture of
*Leishmania mexicana***
التحري عن سمية الميريوسين في المزروع المختبري لطفيلي *Leishmania Mexicana*

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Abstract

Serine Palmitoyltransferase SPT is the key enzyme in the *de novo* sphingolipids biosynthesis pathway in eukaryotes, including the intracellular parasite *Leishmania*. Previous studies showed that this enzyme SPT is expressed only in divided promastigote forms and it is non-essential in the amastigotes form of *Leishmania major*, which is known as the old world leishmaniasis. In this study we have studied the viability of new world leishmaniasis, *Leishmania mexicana*. Cytotoxicity test used to determine the effect of the SPT inhibitor myriocin which did not significantly affect the viability of the two forms of the *in vitro* cultures of the parasite $p > 0.05$, procyclic promastigotes and amastigotes, in which cell viability for miltefosine treatment 100, 50, 25, 12.5, 6.25 μM against procyclic promastigotes form was 133.7, 115.8, 103.7, 94.98, 94.78 respectively for 24 hours incubation, while for amastigotes, cell viability for miltefosine treatment was 114.77, 114.34, 104.21, 132.95, 102.74 respectively for the 24 hours incubation and was 81.46, 81.25, 71.40, 68.38, 66.51 respectively for the 48 hours incubation and was 78.99, 90.22, 85.59, 95.18, 84.43 respectively for the 72 hours incubation. While in the old world leishmaniasis, *Leishmania major*, it has been found that ceramide production is absent and non-essential for the proliferation of intra-amastigotes.

Key words: *Leishmania mexicana*, myriocin, cytotoxicity, SPT.

المستخلص

الانزيم Serine Palmitoyltransferase SPT هو الانزيم الاساسي في البناء الحيوي للدهون الاسفنجولية في الكائنات حقيقية النواة، من ضمنها جنس اللشمانيا الذي يعيش داخل خلايا المضيف . دراسات سابقة اثبتت ان هذا الانزيم موجود في الاطوار المستوطنة و لكنه غير مهم في دورة حياة طفيلي لشمانيا العالم القديم *Leishmania major* . في هذه الدراسة ، اعتمد فحص السمية للتحري عن سمية الميريوسين مثبط انزيم الـ SPT والذي لم يكن لها تاثير على حيوية طفيلي لشمانيا العالم الجديد *Leishmania Mexicana* بطوريه المسوط و عديم السوط. كما ان دراسات سابقة حول لشمانيا العالم القديم *L. major* اثبتت ان انتاج الـ ceramides غير موجود و غير مهم لتضاعف الاطوار عديمة السوط لهذا الطفيلي .

الكلمات المفتاحية : ميريوسين ، السمية ، *Leishmania Mexicana* ، SPT

Introduction

Leishmaniasis species is a genus of different species causes three main health problems known as leishmaniasis; dermal cutaneous leishmaniasis, visceral leishmaniasis and mucocutaneous leishmaniasis and diffused cutaneous leishmaniasis [1]. *Leishmaniasis* is a vector-borne disease, in which the infective form of the parasite, promastigotes, is transmitted through the bite of the female sand fly of the genus *Phlebotomus* old world or *Lutzomyia* new world, to the vertebrate host [2]. With absence of vaccine, new treatments are therefore needed to be found; a potential drug target to be considered and investigated is the intracellular parasite's sphingolipids biosynthesis pathway [3]. Sphingolipids are important membrane compounds and powerful signaling molecules, between five to ten percent of all lipids within the membrane of *Leishmania* are sphingolipids [4]. They are involved in regulating of cell growth, membrane trafficking signal transduction and formation and maintenance of lipid microdomains rafts [5]. Another studies found that the composition, metabolism and function of phospholipids and sphingolipids differ significantly from *Leishmania* than those of mammalian cells [6].

De novo sphingolipids pathway of all eukaryotes starts with the condensation of L-Serine and palmitoyl co-A by the enzyme Serine Palmitoyltransferase SPT, which is considered as the key enzyme of this process which is usually carried out at the endoplasmic reticulum [7].

Ceramide, a subsequent compound in this process, is either transformed into Inositol Phosphorylceramide IPC by the enzyme IPC synthase and this is only found in Fungi, Plants and kinetoplastid including *Leishmania*; while in mammals, Ceramide is transformed into sphingomyelin SM by the enzyme SM synthase, figure (1) [3].

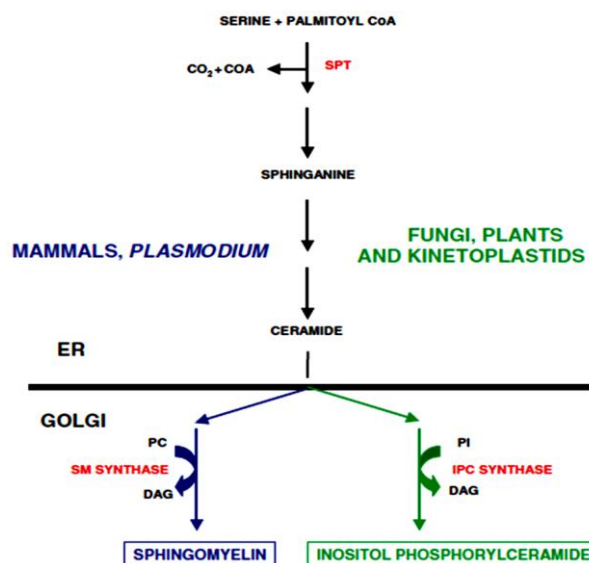


Fig. (1): Dichotomy of Sphingolipids biosynthesis in mammals and kinetoplastids, (ER: Endoplasmic reticulum) [3].

Myriocin, which is a potent inhibitor of the enzyme SPT derived from a thermophilic fungi [8], using this SPT inhibitor 10 μm myriocin found to inhibit the viability of the procyclic forms upon generating the infective metacyclic promastigotes in the old world leishmaniasis *L. major* but did not affect the viable procyclic promastigotes in the log phase, and it had been concluded that the *de novo* sphingolipids is essential for differentiation but not growth of the parasite [4].

In this study, cytotoxicity of myriocin had been investigated against *in vitro* forms of the new world *Leishmania mexicana*, amastigotes and procyclic promastigotes.

Materials and methods

1- Cell culture *L. mexicana* (procyclic promastigotes and amastigotes):

Leishmania Mexicana MYNC/BZ/M379 was kindly provided by Dr Paul Denny, Chemistry department, Durham University, UK.

- Cell culture of procyclic promastigotes was maintained at 26°C in Schneider's *Drosophila* medium Invitrogen at pH7, supplemented with 15% heated inactivated foetal bovine serum HIFBS purchased from FBS, Biosera Ltd [9].
 - Promastigotes were differentiated into amastigotes axenic metacyclogenesis as previously described by [9], in brief, 48 hours culture suspension of promastigotes was transformed into Schneider's *Drosophila* medium containing 20% of HIFBS at pH5.5; promastigotes were seeded at 5×10^5 cell/ml and incubated at 26°C for six days, to produce the metacyclic promastigotes. At day six, the production of amastigotes was induced by transferring the metacyclic promastigotes into a new Schneider's *Drosophila* medium with 20% HIFBS, pH5.5, where cells seeded at 5×10^5 cell/ml and incubated at 32°C for at least five days where cells became amastigotes in culture.
- 2- Myriocin cytotoxicity and Alamar Blue® assay:
- Myriocin cytotoxicity was screened against *L. mexicana* promastigotes and amastigotes using Alamar Blue® quantitative assay and the results were detected using micro-plate reader Biotek® as described by [10 and 11], in brief, two of 96 well-plates flat bottoms were set up for both culture of the suspension of *L. mexicana* promastigotes and amastigotes suspended in Schneider's *Drosophila* medium 15 % HIFBS for promastigotes and 20 % HIFBS for amastigotes, 2 folded serial dilutions were set up to achieve triplicate series of cell culture concentrations between 4×10^5 cell/ml and 1.25×10^4 cell/ml, 100 μl / well.

- Myriocin was prepared according to the manufacturer's procedure of Promega 2 mg powder dissolved in 5 ml methanol then was added to the plates starting with the concentration of 100 μ M and then 2 folded serial dilutions were made to end up with 6.25 μ M.
- Control was prepared the same as above but with no myriocin; absolute methanol was added instead myriocin solvent.
- samples were set up as triplicates for test and control samples and the florescent absorption was detected by micro-plate reader Biotek[®] after 24 hours incubation at 26^oC for promastigotes and 24, 48 and 72 hours at 32^oC for amastigotes.
- T-test was used for cytotoxicity results analysis; p value was to be significant when p<0.05.
- Myriocin cytotoxicity was screened against axenic culture of promastigotes and amastigotes of *L. mexicana* and the viability percentage was calculated according to the following equation:

$$\text{- Percentage of viability} = \frac{\text{Plate-absorption reading of each test triplicate}}{\text{Mean of plate reading of control triplicate}} \times 100$$

Results and discussion

1- Cytotoxicity of myriocin against promastigotes:

Results were plotted against log myriocin concentrations.

Promastigotes were grown normally, with no cytotoxic effect of myriocin and the proliferation of the parasite was normal in the presence of myriocin. Statistically, there was no significant difference in plate's fluorescent absorption between test and control for concentrations of 6.25, 12.5, 25 μ M but there was significant difference p < 0.05 at concentrations of 50 and 100 μ M in which colorimetric absorption was higher in treated sample and cell viability was no less than 94.78 % for all concentrations figure (1).

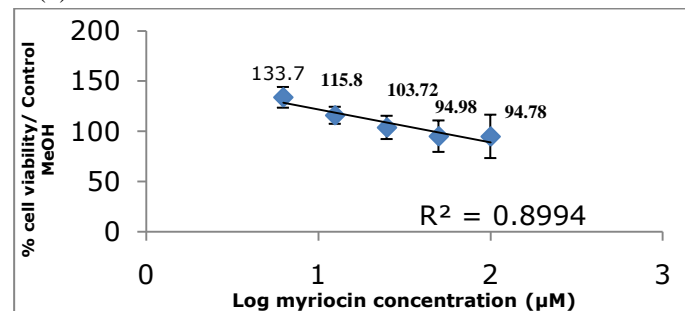


Fig. (1): Cell viability of *L. mexicana* promastigotes treated with myriocin.

2- Cytotoxicity of myriocin against amastigotes:

Results showed that amastigotes were grown normally in all incubation periods 24, 48, 72 hours and the myriocin did not affect the proliferation of the parasites. Plate of 24 hours incubation showed significant difference p < 0.05 in colorimetric absorption at concentration of 12.5 and 50 μ M but the percentage of viability was 102.74% as in figure (2), while plate of 48 hours incubation showed significant difference at all concentrations but the percentage of viability was no less than 66.5 % Figure (3) and the plate of 72 hours incubation showed significant difference p < 0.05 at concentrations of 6.25 and 100 μ M but the percentage of viability was no less than 78.99 % as in figure (4).

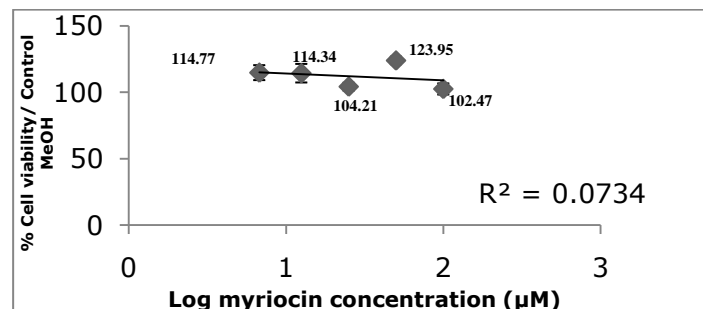


Fig. (2): Cell viability of *L. mexicana* amastigotes treated with myriocin, after 24 hours incubation.

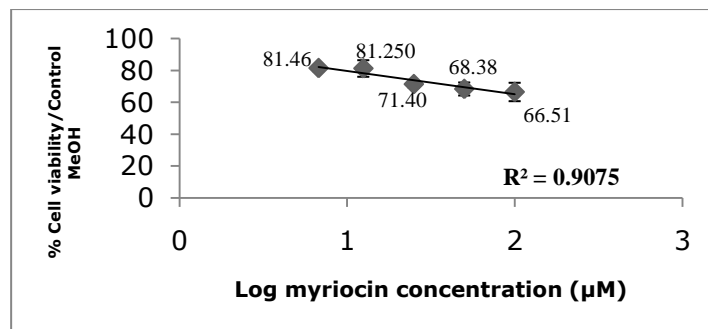


Fig. (3): Cell viability of *L. mexicana* amastigotes, after incubation with myriocin, after 48 hours incubation.

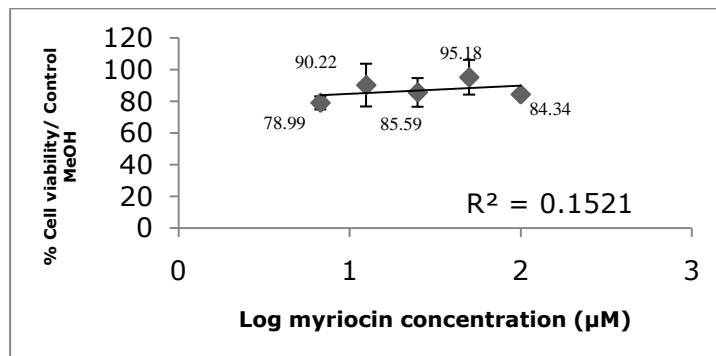


Fig. (4): Cell viability of *L. mexicana* amastigotes, treated with myriocin, after 72 hours incubation.

Results of the viability percentage showed that myriocin, the potent inhibitor of the enzyme SPT, had a minor cytotoxic effect on the proliferation of the two forms of the axenic culture of the parasite, in which test fluorescent absorption was relatively similar to the controls. Although some significant difference was noticed in which the treated cells had a viability percentage more than the untreated cells. These results can be concluded with the findings of a previous study [12, 13] as they found that treating mouse macrophage cell line RAW264.7 with myriocin or other potent inhibitors of SPT inhibited the accumulation of ceramide in these cells; such ceramide is emerged as an important second messenger that may mediate a number of biological processes including induction of cell death [14]. While the accumulation of ceramide in these cells found to be involved in the propagation caspase-dependent macrophage cell death [13].

However, treating the two forms of the axenic culture of *L. mexicana* did not affect the proliferation and the viability of the parasite, as all viability percentage of cultures were more than 50 % for all concentrations of myriocin along the different incubation periods.

Previous study on the wild type old world *L. major* treated with 10 µM myriocin did not limit the growth of the parasite but inhibited the synthesis of both Inositol phosphorylceramide IPC and ceramides, detected by thin layer chromatography TLC [4]. Further to understand the role of *de novo* sphingolipids biosynthesis in *L. major*, another study created sphingolipids null mutant of SPT subunit two *spt2* of this old world *Leishmania* species, also treated with 10 µM myriocin, results showed that the IPC and ceramides synthesis were also inhibited and upon entry to stationary phase, the *spt2* promastigotes of *L. major* failed to differentiate and died instead [14].

Also another study [15] showed that in mouse model, the intra-amastigotes derived from infected macrophages of mouse lesion maintained IPCs at the high of wild type level, concluding that this parasite has an efficient mechanism for acquiring host metabolites necessary for IPC synthesis. Also they found that in the mouse model, the amastigotes of *L. major* found to synthesize IPC without SPT and are likely to scavenge ceramide from the host (mouse) for use as IPC synthase substrate.

Most recent study on the new world leishmaniasis, *L. mexicana*, found that the SPT is expressed in both stages of the axenic culture of promastigotes and amastigotes [16], the study found that *Leishmania mexicana* SPT subunit two (*LmxLCB2*) and so *LmxSPT* protein is expressed in the two forms of *L. mexicana* concluding that the viability of the parasite after myriocin treatment can be

related to the ability of the *de novo* sphingolipids biosynthesis by the parasite. Also, a proteomic analysis of *L. mexicana* differentiation [17] found that one of the protein identified from the parasite is SPT.

On the light on the available results and data by other studies, we can conclude that the viability of the axenic culture of *L. mexicana* treated with the SPT inhibitor, myriocin, did not inhibit the proliferation of the parasite and had a minor effect on the parasite and this is due to the ability of this new world *Leishmania* species to express the SPT in both *in vitro* stages (procyclic promastigotes and amastigotes) and this is one of the differences between the old and new world *Leishmania*, as in the old world *L. major*, the *Lmj*LCB2 is down-regulated and non-essential for pathogenesis [3 and 4].

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