Biochimica et Biophysica Acta 1859 (2017) 1–9

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The cytotoxic activity of miltefosine against *Leishmania* and macrophages is associated with dynamic changes in plasma membrane proteins

Kelly Souza Fernandes [a,b], Paulo Eduardo Narcizo de Souza [c], Miriam Leandro Dorta [d], Antonio Alonso [a,*]

[a] *Instituto de Física, Universidade Federal de Goiás, Goiânia, GO, Brazil*
[b] *Instituto Federal Goiano, Urutaí, GO, Brazil*
[c] *Instituto de Física, Universidade de Brasília, Brasília, DF, Brazil*
[d] *Instituto de Patologia Tropical e Saúde Publica, Departamento de Imunologia e Patologia Geral, Universidade Federal de Goiás, Goiânia, GO, Brazil*

ARTICLE INFO

Article history:
Received 23 June 2016
Received in revised form 16 September 2016
Accepted 17 October 2016
Available online 20 October 2016

Keywords:
Miltefosine
Leishmania
Macrophages
Membrane fluidity
Protein dynamics

ABSTRACT

In this study, we combined electron paramagnetic resonance (EPR) spectroscopy with an analysis of biophysical cellular parameters to study the mechanisms underlying the *in vitro* anti-leishmanial activity of miltefosine (MT). A thiol-specific spin label attached to membrane-bound proteins of *Leishmania amazonensis* and peritoneal macrophages indicated that MT may bind to plasma membrane proteins in large quantities via a detergent-like action and cause structural changes associated with a marked increase in dynamics and exposure to an aqueous environment. EPR spectra of a spin-labeled stearic acid indicated strong interactions between the probe and membrane proteins and a marked increase in the membrane fluidity of MT-treated cells. The cytotoxicity of MT was found to depend on the cell concentration used in the assay. This dependence was described by an equation involving the 50% inhibitory concentrations of MT in the aqueous medium (c_{w50}) and the cell membrane (c_{m50}) and the membrane-aqueous medium partition coefficient of MT (K). With a c_{w50} of 8.7 μM, macrophages were less sensitive to MT than amastigotes and promastigotes of *Leishmania*, which had c_{w50} values of 2.4–3.1 μM. The estimated c_{m50} of MT for *Leishmania* was 1.8 M, which appears sufficient to cause ruptures or formation of pores in the plasma membrane. Additionally, we demonstrated that the changes in the plasma membrane detected by EPR spectroscopy occurred at cytotoxic concentrations of MT, as assessed through *in vitro* assays.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Miltefosine (MT) is an oral drug approved for the treatment of leishmaniasis in India (2002), Germany (2004), Colombia (2005) and the United States (2014, for patients aged ≥12 years) [1]. It has also been used to treat fatal granulomatous amebic encephalitis [2] and as a topical treatment for breast cancer skin metastases and cutaneous lymphoma [3]. MT also exhibits antibacterial [4] and broad-spectrum antifungal activities [5,6]. Although its mechanism of action has not yet been well established, knowledge of this mechanism could allow the rational design of more potent and less toxic analogs [6]. The anticancer mechanisms of MT were recently revised. At higher concentrations, the detergent properties of MT cause cell lysis, whereas at lower and more clinically relevant concentrations, this drug causes only biophysical disturbances in the cell membrane, thereby interfering with phospholipid turnover, lipid-based signal transduction pathways and membrane microdomain formation [7]. Interestingly, the major molecular targets of the anticancer activity of MT have been suggested to also be anti-leishmanial targets, including the inhibition of phosphatidylcholine biosynthesis and the induction of apoptosis through inhibition of the PI3K/Akt/PKB pathway [7,8].

EPR spectroscopy of spin labels was recently employed to demonstrate that MT does not essentially alter the lipid dynamics in model membranes [9,10]. However, MT markedly increases the membrane fluidity of *Leishmania* [11] and erythrocytes [10], suggesting that it acts preferentially on membrane proteins. However, EPR experiments require samples with a small volume (~30 μL) and high cell concentrations (minimum of 5×10^8 cells/mL), and as a result, membrane alterations have been observed only in the presence of approximately 1 mM MT [11]. This concentration is considerably higher than the typical IC_{50} values of MT, particularly because anti-proliferative assays are not performed under physiologically relevant conditions. Approximately 5×10^9 RBCs/mL are present in the blood, but the typical cell concentrations used for *in vitro* experiments are approximately one thousand times lower. The important biophysical parameters discussed in this study must be considered in anti-proliferative activity assays conducted under such dilute conditions.

The IC_{50} of MT for *Leishmania* was recently found to increase with increases in the experimental cell concentration [11]. One of the objectives of this study was to elucidate the biophysical parameters involved in the relationship between the drug's cytotoxic capacity and

* Corresponding author.
E-mail address: alonso@ufg.br (A. Alonso).

http://dx.doi.org/10.1016/j.bbamem.2016.10.008
0005-2736/© 2016 Elsevier B.V. All rights reserved.

the cell concentration used in the anti-proliferation assay. To accomplish this, we investigated the dependence of the IC_{50} of MT for *L. amazonensis* (axenic amastigotes and promastigotes) and peritoneal macrophages on the cell concentration. Additionally, we demonstrated that the MT-induced changes in *Leishmania* and the macrophage membranes detected by EPR spectroscopy occur in the same cytotoxic drug concentration range.

2. Materials and methods

2.1. Chemicals

The spin labels 5-doxyl-stearic acid (5-DSA) and 4-maleimido-1-oxyl-2,2,6,6-tetramethylpiperidine (6-MSL) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and MT was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

2.2. Cells

Promastigotes and amastigotes of *L. (L.) amazonensis* (MHOM/BR/75/Josefa) reference strains were grown in 24-well microtiter plates containing 2 mL of Grace's insect medium (Sigma-Aldrich) supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 100-U/mL penicillin and 100-μg/mL streptomycin (Sigma-Aldrich), as previously described [11]. Axenic *L. amazonensis* amastigotes were grown as described previously [12]. As reported, this form has been used because its morphology, structure and infectivity are similar to those of intracellular amastigotes (and are different from those of promastigotes) [12–14]. In contrast to the *in vitro* process used to obtain parasites directly from lesions, it is possible to obtain higher concentrations of amastigotes and eliminate possible contamination by elements of vertebrate hosts. These forms were grown as described previously [11], and tests were performed once these reached the logarithmic phase of growth (6th day). Peritoneal macrophages were obtained from BALB/c mice as previously reported [15] in accordance with a research project approved by the Ethics Committee of Human and Animal Medical Research at the Hospital das Clínicas da Universidade Federal de Goiás (125/2004) and cultured in RPMI-1640 medium supplemented with 10% FCS.

2.3. In vitro anti-proliferative activity assays

Parasites or macrophages at several cell concentrations were treated with increasing concentrations of MT diluted in culture medium supplemented with 10% FCS for 24 h or without FCS for 2 h (Corning Life Sciences, Corning, NY, USA) and incubated in 96-well culture dishes (100 μL for parasites and 200 μL for macrophages). Cell viability was assessed by measuring the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) by metabolically active cells, as described previously [11]. Measurements were performed in triplicate for each treatment, and the values obtained were used to calculate the mean percentage of viable cells relative to the control. The IC_{50} was then determined by fitting the concentration response with a sigmoid curve.

2.4. Spin labeling and MT treatment

To incorporate the lipid spin label 5-DSA into the *Leishmania* or macrophage membranes, a spin label film was first prepared on the bottom of a test tube, as described previously [11]. A 1-μL aliquot of a stock solution of 5-DSA in ethanol (4 mg/mL) was transferred to a glass tube, and after solvent evaporation, 50 μL of a cell suspension in Grace's medium was added to the spin-label film, followed by gentle agitation. Spin-labeling of the *Leishmania* membrane proteins was performed by incubating a cell suspension containing 2 mM 6-MSL for 2 h at 26 °C. To remove the free spin label, the sample was centrifuged (5,000 $\times g$, 4 °C) for 15 min and then resuspended in Grace's medium (this procedure was repeated eight times). After spin labeling, a similar procedure was applied for the treatment of cells with MT [11]. An aliquot (1–5 μL) of a stock solution of MT in ethanol (3 or 10 mg/mL) was used to generate films in glass tubes with MT amounts varying from 30 to 300 μg. For the EPR measurements, the samples were transferred to 1-mm-i.d. capillary tubes, which were sealed using a flame after labeling and treatment.

2.5. Membrane disruption measurements

Leishmania amastigotes and macrophages spin-labeled with 6-MSL at cell concentrations of 2×10^9 parasites/mL and 7×10^7 macrophages/mL were treated with MT in Grace's medium without FCS. After a 2-h incubation, the samples were centrifuged at 15,000 $\times g$ for 5 min, and the EPR spectra of the supernatants were recorded to assess the amounts of spin-labeled membrane fragments. In parallel, the protein content of the supernatants was measured using a commercial kit (Sigma) based on the bicinchoninic acid (BCA) reaction.

2.6. EPR spectroscopy

A Bruker EMX Plus spectrometer (Rheinstetten, Germany) equipped with an ER4102ST cavity was used to perform the EPR measurements. Spectra were acquired using the following instrumental settings: microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; magnetic field scan, 100 G; sweep time, 168 s; and sample temperature, 25 °C.

2.7. Cell viability assay

L. amazonensis amastigotes and peritoneal macrophages were treated with MT as in the EPR experiments, and the cell viability was assessed by counting the live cells under a microscope using the vital stain Trypan Blue (Aldrich). Briefly, samples of culture medium supplemented with 10% FCS containing 2×10^9 amastigotes/mL or 7×10^7 macrophages/mL were treated with MT. After 5 min of incubation, the samples were diluted 40-fold, and 0.1% (w/v) Trypan Blue (Sigma-Aldrich) was added. The living cells in each sample were counted in a Neubauer chamber.

2.8. Statistical analysis

All data are presented as the means ± S.D.s from at least three independent experiments. The data were compared through one-way analysis of variance (ANOVA) followed by Tukey's multiple range test, and differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. The cytotoxicity of MT depends on the cell concentration

Table 1 shows the IC_{50} values of MT found for several *Leishmania* and macrophage concentrations and indicates that lowering the cell concentration in the assay also significantly decreased the IC_{50} value. Identification of the biophysical parameters that modulate the cytotoxic concentration of a hydrophobic drug in a cell suspension is relevant to any trial involving drug-cell interactions.

If n_w moles of the drug are present in aqueous medium and n_m moles are present in the cell membrane, the molar concentration in the suspension (c_{sus}) is given by

$$C_{sus} = \frac{n_w + n_m}{V_w + V_m}, \tag{1}$$

where V_w is the volume of aqueous medium, and V_m is the membrane volume of the cells in the suspension. Introduction of the molar

Table 1
Effects of MT against amastigotes and promastigotes of *L. amazonensis* and peritoneal macrophages from BALB/c mice (MTT assay)

Cells/mL	IC_{50} (μM) 24 h – 10% FCS	2 h – without FCS
Amastigotes		
1×10^8	54 ± 8 (A)[a]	55 ± 6 (A)
4×10^7	35 ± 5 (B)	24 ± 1 (B)
2×10^7	23 ± 4 (BC)	11 ± 2 (C)
5×10^6	13 ± 2 (C)	6 ± 1 (C)
Promastigotes		
1×10^8	59 ± 9 (A)	59 ± 11 (A)
4×10^7	43 ± 9 (AB)	24 ± 5 (AB)
2×10^7	26 ± 6 (BC)	20 ± 6 (BC)
5×10^6	12 ± 5 (C)	9 ± 4 (C)
Macrophages		
1×10^7	238 ± 4 (A)	96 ± 3 (A)
5×10^6	233 ± 9 (A)	63 ± 2 (B)
$2,5 \times 10^6$	–	33 ± 2 (C)
1×10^6	146 ± 3 (B)	24 ± 2 (D)
5×10^5	137 ± 15 (B)	14 ± 1 (E)

[a] Statistical significance: for each column and cell type, the data that do not have capital letters in common are significantly different at $P < 0.05$.

concentrations in the membrane (c_m) and the aqueous phase (c_w) and the cell membrane-water partition coefficient (K, where $K = c_m/c_w$) into Eq. (1) yields

$$c_{sus} = \frac{c_w V_w + K c_w V_m}{V_w + V_m} \tag{2}$$

or

$$c_{sus} \left[\frac{(V_w/V_m) + K}{(V_w/V_m) + 1}\right] c_w. \tag{3}$$

The value of the term in brackets tends equal 1 in well-diluted systems ($V_w \gg V_m$) or for hydrophilic drugs (low values of K). In these cases, $c_{sus} = c_w$. However, for hydrophobic molecules and higher cell concentrations, the suspension can no longer be considered homogeneous, and c_{sus} can be quite different from c_w.

To estimate the membrane volume of each cell (V_{mc}), we assumed an ellipsoidal shape for the amastigotes and promastigotes of *Leishmania* and a spherical shape for macrophages and measured the major and minor axes of the parasites and the diameter of the macrophages (Table 2). The volume and surface area of the cells were calculated, and as explained below, a cell membrane thickness of 78 Å was used to assess the V_{mc}. In a 1-mL suspension, the V_m is equal to the V_{mc} multiplied by the number of cells per mL (c_c), and the V_w closely approximates 1 mL, i.e., the suspension volume (because the value of V_m is very small, varying from 4.1×10^{-6} to 1.6×10^{-3} mL for the smallest

Table 2
Dimensional measurements of *Leishmania* and peritoneal macrophages (n = 100) and calculated values of the volume, surface area, and cell membrane volume (V_{mc})

Cell parameters	*Leishmania* Amastigotes	Promastigotes	Macrophages
Major axis (μm)	4.3 ± 0.7	10.0 ± 2.0	
Minor axis (μm)	3.1 ± 0.3	4.0 ± 1.0	
Diameter (μm)			13.0 ± 1.0
V_c (cell volume, fL)	21.6	83.8	1150
Surface area $(\mu m)^2$	38.2	104.5	531
V_{mc} (10^{-13} mL)	2.98	8.17	41.4

to the greatest cell concentration used, respectively). Therefore, we can write an equation for c_{sus} as a function of the cell concentration:

$$c_{sus} = \left[\frac{(V_{mc} \cdot c_c)^{-1} + K}{(V_{mc} \cdot c_c)^{-1} + 1}\right] c_w. \tag{4}$$

Fig. 1 shows graphs of the IC_{50} data (expressed as c_{sus}) presented in Table 1 for various cell concentrations. The best-fit curves produced by Eq. (4) gave estimated values for K and c_w in the cell suspension (Table 3). The c_w values found can be designated as c_{w50} values because they represent the MT concentration in the aqueous phase of the cell suspension that results in 50% cell death (the c_{m50} values were calculated using $K = c_{m50}/c_{w50}$). Importantly, unlike the IC_{50}, the c_{w50} and c_{m50} values are independent of the cell concentration, indicating that these cell-suspension parameters are adequate for comparing the effects of MT on different cell types. In fact, the theoretical curve fitting the experimental data using Eq. (4) also allows estimation of V_{mc} (the values of K, c_w and V_{mc} affect the slope and the vertical and lateral displacements of the curve, respectively), and consequently, the membrane thickness since the cell dimensions were measured. The estimated membrane thickness of 78 Å coincides with the value reported for the erythrocyte membrane [16].

In the presence of FCS, the c_{w50} values for amastigotes of *Leishmania* and macrophages were approximately 5- and 15-fold higher, respectively, indicating that the FCS retains some MT due to the binding of MT to the albumin [8] present in the FCS, thereby decreasing the MT that reaches the cells. The K value indicated that the affinity of MT for the *Leishmania* membrane was markedly higher than that for the erythrocyte membrane. However, the critical concentration that causes disruption of the erythrocyte membrane was also markedly lower than the c_{m50} of *Leishmania* (Table 3). Macrophages were found to be less sensitive to MT than *L. amazonensis*. In addition, the c_{m50} value of the macrophages was significantly higher in the experiment using FCS and a 24-h incubation period. We suspect that some of the MT entered the macrophages during this longer incubation period, and as a result, the effective concentration of MT in the plasma membrane was actually less than the calculated value.

High cell concentrations are necessary to obtain good spectra in EPR spin label experiments. In this study, samples containing 2×10^9 parasites/mL or 7×10^7 macrophages/mL were used. Based on the c_{w50} and K values shown in Table 3, the corresponding IC_{50} values of MT for these cell concentrations were calculated (using Eq. (4)) as 1.1 mM for *Leishmania* and 0.7 mM for macrophages.

Fig. 2 shows EPR spectra of spin-labeled plasma membranes of *Leishmania* amastigotes and macrophages. The spectral parameters $2A_{//}$ of the spin labels 5-DSA (lipid component) and 6-MSL (protein component) gradually decreased as the MT concentration increased, indicating that the membrane fluidity of both cell types increased substantially. For 0.7 mM MT, the lowest experimental concentration used, the mean $2A_{//}$ values of both spin labels in the two cell types showed small reductions that were only slightly greater than the estimated experimental error (0.5 G). However, for the second highest concentration (1.4 mM MT), the average values of $2A_{//}$ were significantly lower than those of the control samples ($P < 0.05$). Notably, a gradual increase in the MT concentration to ~20 mM generated a disproportionate decrease in the $2A_{//}$ values, consistent with protein denaturation. This finding indicates that MT can bind to membrane proteins in large quantities.

EPR spectroscopy associated with site-directed spin labeling has been widely used for the assessment of the dynamics and structural topology of proteins [18]. The EPR spectra of spin-labeled proteins are generally composed of two spectral components resulting from two spin-probe populations with distinct mobility, which are known as strongly (S) and weakly (W) immobilized components [11]. The more mobile component (W), which is indicated by three sharp resonance lines, was predominant in the EPR spectra of 6-MSL for macrophage

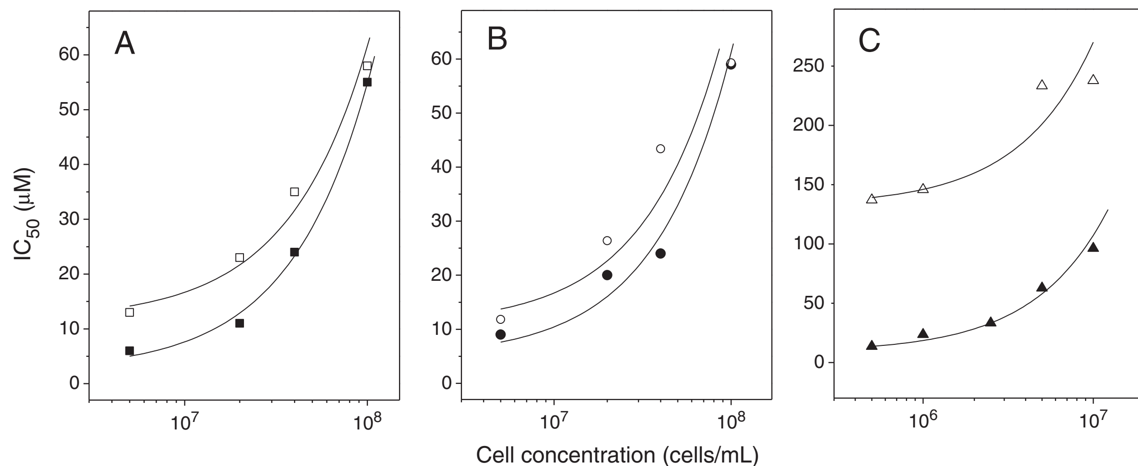

Fig. 1. IC_{50} values of MT in amastigotes (A) and promastigotes (B) of *Leishmania* and macrophages (C). These values were calculated from data presented in Table 1 for several cell concentrations with best-fit curves based on Eq. (4). The experiments were performed in culture medium with fetal calf serum (FCS) and a 24-h incubation period (open symbols) or in culture medium without FCS and a 2-h incubation period (closed symbols).

membranes (Fig. 2E). The less mobile component (S), which exhibits a broad spectral line shape, was predominant in the EPR spectra for the parasite membranes (Fig. 2D). In Fig. 2E, the first and last resonance lines of each spectrum, which include contributions from component S only, are amplified 30-50 times to facilitate better visualization of their magnetic field positions. The S component of this thiol-reagent spin label, which was bound to the *Leishmania* and macrophage membranes, had $2A_{//}$ values of approximately 66 G, similar to those for erythrocyte membranes [10] and bovine serum albumin (BSA) spin labeled at its single sulfhydryl group [19]. For macrophages, unlike *Leishmania* and erythrocytes [10], a considerably smaller S-component fraction was observed in the spectra (Fig. 2E), indicating that 6-MSL reacted predominantly with SH groups in cavities that were more open and exposed to the aqueous solvent. Moreover, the S-component fraction also decreased gradually as the MT concentration in the cell suspension increased, suggesting that MT can bind to membrane proteins in amounts that are sufficient for denaturation.

To determine whether the EPR spectra could detect changes in the membrane fluidity of *Leishmania* caused by MT in a commonly reported IC_{50} range for cytotoxicity assays, we performed an EPR experiment using samples with a lower parasite concentration (4×10^7/mL) and a greater suspension volume. After treatment with MT and a 2-h incubation, the samples were centrifuged to increase the cell concentration 40-fold. The resultant EPR spectra, which are shown in Fig. 3, indicate that 15 µM MT is sufficient to increase the membrane fluidity of *Leishmania*.

Table 3
Biophysical parameters associated with MT activity in the cell membranes of amastigotes and promastigotes of *L. amazonensis*, peritoneal macrophages, and erythrocytes

Cells	K (10^5)[a]	log K	c_{w50} (µM)	c_{m50} (M)
Assay with FCS (24 h)				
Amastigotes	1.44 ± 0.15 (A)[b]	5.16	12 ± 2 (A)	1.7 ± 0.2 (A)
Promastigotes	0.68 ± 0.02 (B)	4.83	11 ± 3 (A)	0.7 ± 0.2 (B)
Macrophages	0.25 ± 0.02 (C)	4.40	132 ± 8 (B)	3.3 ± 0.4 (C)
Assay without FCS (2 h)				
Amastigotes	7.40 ± 1.47 (D)	5.87	2.4 ± 0.3 (C)	1.8 ± 0.3 (AD)
Promastigotes	2.41 ± 2.06 (E)	5.38	3.1 ± 0.5 (C)	0.7 ± 0.3 (B)
Macrophages	2.85 ± 0.69 (E)	5.45	8.7 ± 2.1 (A)	2.5 ± 0.4 (D)
Erythrocytes[c]	0.48	4.68	2.3	0.11

[a] The best-fit parameters were obtained using Eq. (4) and the data presented in Fig. 1: K, membrane-culture medium partition coefficient; c_{w50} and c_{m50}, MT concentrations in the aqueous phase and membrane, respectively, that kill 50% of cells.
[b] Statistical significance: in each column, the data that are not shown with the same capital letter are significantly different at $P < 0.05$.
[c] The data reported in ref. [17] for 50% erythrocyte hemolysis in phosphate-buffered saline (PBS) are shown for comparison.

3.2. MT induces cell lysis

After the EPR measurements using the spin label 6-MSL, the samples were centrifuged, and an EPR spectrum of the supernatant of each sample was recorded searching for spin-labeled membrane fragments. The EPR spectrum intensity increased as the MT concentration in the samples increased (Fig. 4), indicating that cell lysis occurred. Cell lysis measurements based on the protein content of the supernatant were also performed under the same conditions as those used for the EPR experiments (Fig. 4C). Notably, the changes in membrane fluidity and the cell lysis process are initiated at similar concentrations of MT.

3.3. MT affects cell viability 5 min after treatment

Cell viability measurements were performed using the same cell concentrations utilized for the EPR and cell lysis experiments; however, the culture medium was supplemented with FCS, and the period after treatment with MT was approximately 5 min instead of 2 h (Fig. 5). We want to emphasize that the effect of the FCS decreases at higher cell concentrations, such as those used in this study. Thus, the IC_{50} values for *L.* amastigotes with or without FCS were no longer significantly different when a concentration of 1×10^8 parasites/mL was used (Table 1). As mentioned previously, the cell concentrations used were 2×10^9 amastigotes/mL and 7×10^7 macrophages/mL, and the calculated MT IC_{50} values were 0.7 mM for macrophages and 1.1 mM for *Leishmania* amastigotes. In this experiment, the 50% viabilities were found at 0.7 mM MT for macrophages and 1.8 mM MT for *L.* amastigotes (Fig. 5). This finding indicates that for amastigotes, a 5-min period was insufficient for complete distribution of MT in the membrane. In this case, even with a probable flippase protein activity, MT could be predominantly located in the outer leaflet of the plasma membrane.

4. Discussion

Previous studies have demonstrated that the EPR spectra of 5-DSA in extruded vesicles of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and MT do not change for MT molar ratios of up to 35% [9]. Interestingly, a $2A_{//}$ value of 54.7 G has been found for vesicles of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) containing 30 mol% cholesterol, and the replacement of 20 mol% of the unsaturated lipid POPC with 20 mol% MT yields a $2A_{//}$ value of 55.6 G, indicating a

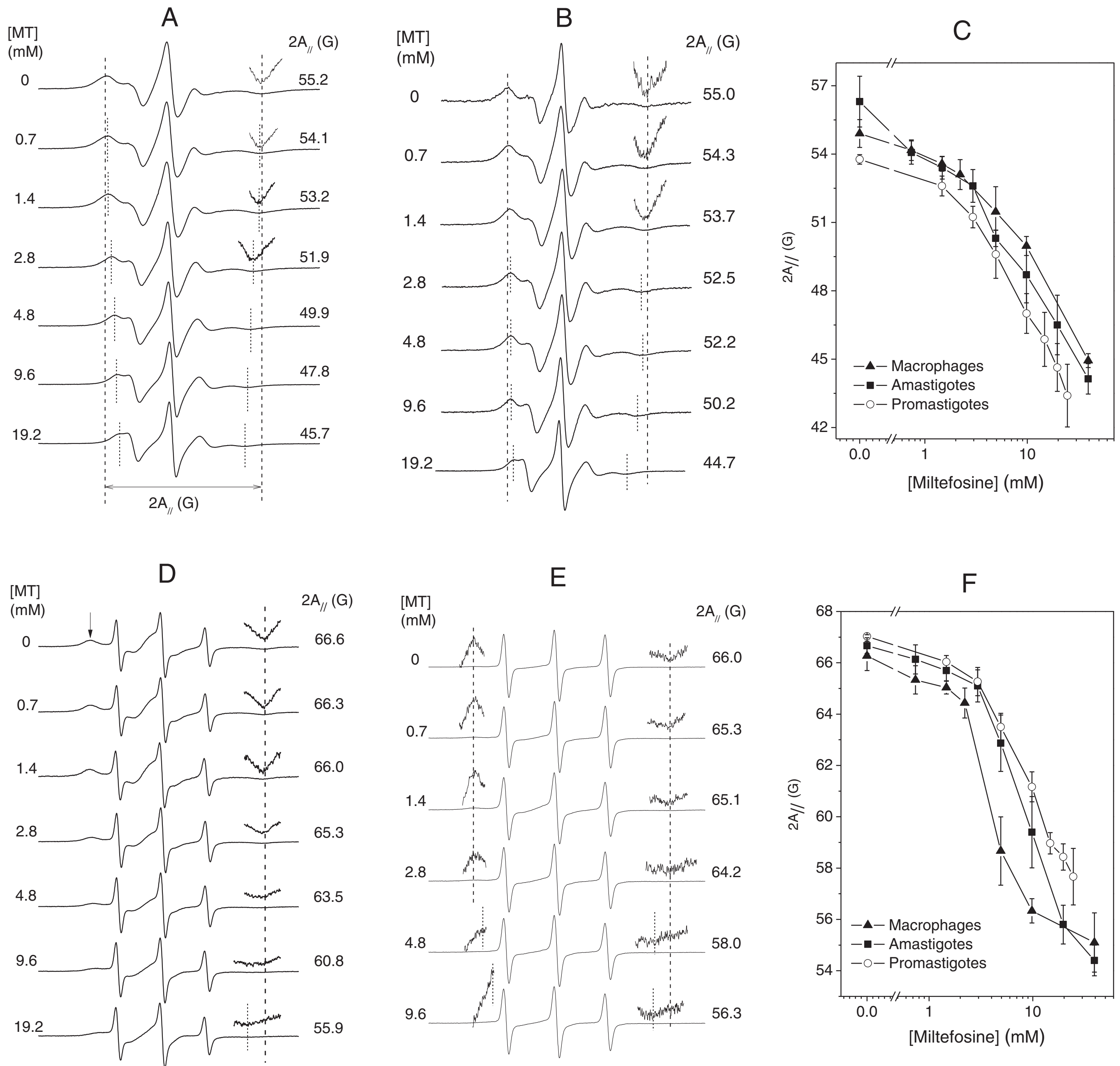

Fig. 2. EPR spectra of the spin label 5-DSA inserted into the plasma membrane of *Leishmania* amastigotes (A) and macrophages (B) that were treated and not treated with several MT concentrations. The values of the EPR parameter $2A_{//}$ (outer hyperfine splitting), which is given by the separation (in magnetic field units) between the first peak and the last inverted peak of the spectrum, are indicated. Some peaks are amplified to clearly indicate the magnetic-field positions, which are indicated by vertical lines. The total magnetic field scan range of each spectrum is 100 G. Panels (D) and (E) show the EPR spectra of 6-MSL covalently bound to the membrane proteins of *Leishmania* amastigotes (D) and macrophages (E). The dependence of the $2A_{//}$ parameter on the MT concentration is plotted for 5-DSA (panel C) and 6-MSL (panel F). Data reported in a previous study [11] for promastigotes of *L. amazonensis* are also shown for comparison.

more rigid lipid bilayer in the presence of MT [10]. Thus, the increases in cell membrane fluidity caused by MT in *Leishmania* and macrophages (Fig. 2C) and erythrocytes [10] and the marked changes in the $2A_{//}$ values of 5-DSA from approximately 56 to 46 G indicate that MT interacts strongly with membrane proteins. For instance, in the stratum corneum (the uppermost skin layer), where some of the extracellular lipids are covalently bound to proteins from the corneocyte envelope, the $2A_{//}$ of 5-DSA decreased from ~63 to ~53 G in the presence of MT [9]. In contrast, in vesicles prepared to mimic the lipid composition of the stratum corneum using commercial lipids (bovine brain ceramide, behenic acid, and Chol at a molar ratio of 2:1:1), a markedly lower value of $2A_{//}$ (~56 G) was observed, and the addition of 20 mol% MT caused only a modest increase in lipid fluidity [9].

EPR spectroscopy has identified two populations of fatty acid spin labels in cell membranes with distinct molecular dynamics [20]. The less mobile spectral component is associated with the boundary lipids in direct contact with the hydrophobic surface of the proteins, whereas the more mobile spectral component is attributed to the spin labels located in the bulk bilayer phase [20,21]. The EPR spectra of 5-DSA in plasma membranes of *Leishmania* amastigotes (Fig. 2A), macrophages (Fig. 2B), *Leishmania* promastigotes [11] and erythrocytes [10] show only the spectral component corresponding to spin labels on the protein

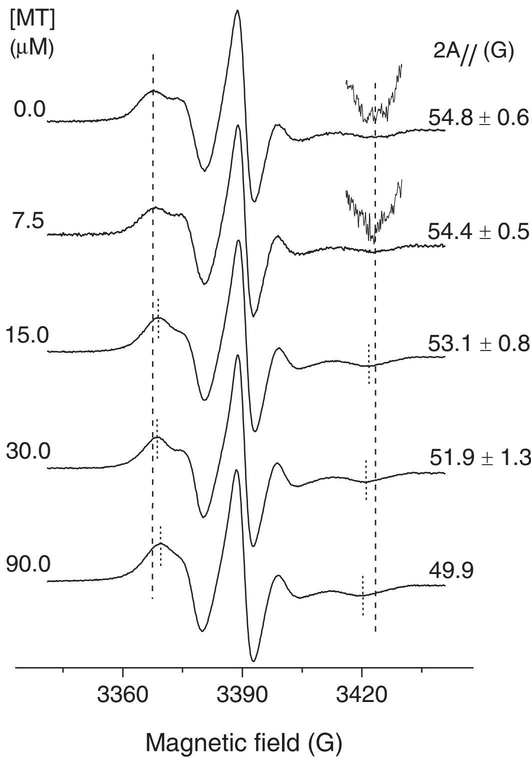

Fig. 3. EPR spectra of 5-DSA in the plasma membrane of *Leishmania* amastigotes untreated and treated with several MT concentrations. The vertical lines indicate the magnetic field positions used in the $2A_{//}$ measurements.

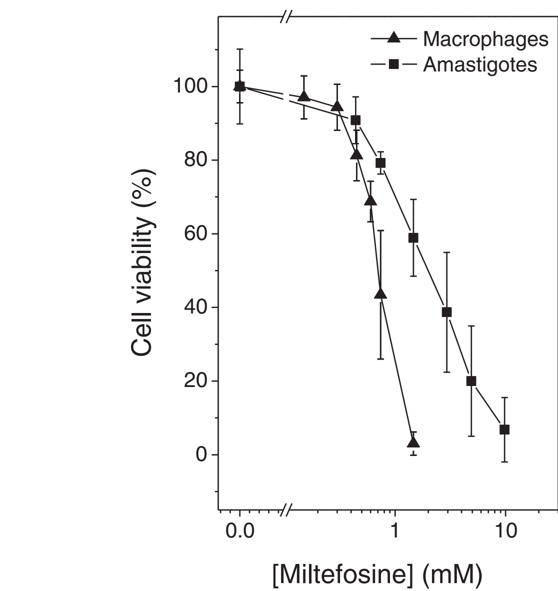

Fig. 5. *Leishmania* amastigote and macrophage viability after treatment with MT. The cell viability was evaluated using the Trypan Blue exclusion method.

boundary layer, probably due to the relatively high protein contents of these membranes. Therefore, we monitored the membrane-bound protein dynamics using two types of probes: one in the lipid component to monitor the dynamics of the intramembranous surface of the proteins and another that was covalently bound to sulfhydryl groups to evaluate the backbone dynamics of the proteins. The spin label 5-DSA has been used to analyze the effects of galactofuranose derivatives on the *L. donovani* promastigote membrane, and MT was used as a reference molecule [22]. Parasites incubated for 48 h and then spin labeled showed significantly more rigid membranes when treated with MT or two other compounds. Rigidifying effects on cell membranes have been associated with lipid peroxidation [23,24].

Spin-label EPR data show that MT can interact strongly with the hydrophobic surfaces of membrane proteins and induce structural changes in a concentration-dependent manner that lead to more dynamic and solvent-exposed conformations. In a previous work [11], the interaction of MT with *L. amazonensis* promastigote membrane proteins was examined with the spin label 5-MSL and by simulating the EPR spectra. In the above-mentioned work, the addition of MT decreased the fraction of the spectral component S in the spectrum, indicating an increased opening of the thiol group cavity and thereby increased solvent exposure. A similar phenomenon was also observed for *L. amazonensis* amastigotes and peritoneal macrophages

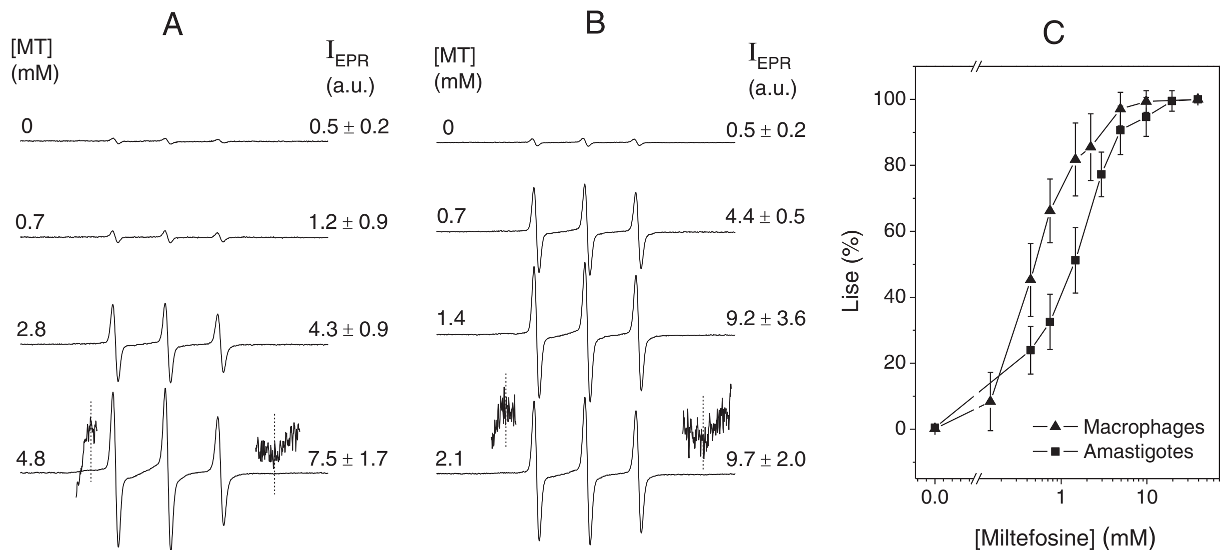

Fig. 4. EPR spectra of the spin label 6-MSL in *Leishmania* (A) and macrophage (B) membranes. The amount of spin-labeled membrane fragments in the supernatant increases with increases in the MT concentration used in the treatment. (C) Percentages of cell lysis based on the protein contents in the supernatants of the same samples.

in this study and for erythrocytes in a previous study [10]. In addition, the effects of MT on membrane-bound proteins, which are characterized by marked increases in molecular dynamics and reductions in the component-S fraction, are similar to those of surfactants on BSA, as assessed by EPR spectroscopy of the spin label 5-MSL attached to the single thiol group of the Cys-34 BSA residue [19].

A mode of action for MT based on the unspecific attack of biological membrane proteins is consistent with the broad-spectrum activity of MT against pathogenic fungi [5,8], *Leishmania* species [10], *Trypanosoma cruzi* [25], granulomatous amebic encephalitis [2], *Streptococcus pneumoniae* [4] and various types of tumor cells [3,9]. However, the potency of cell injury should vary among different cells. Indeed, we found that peritoneal macrophages with a c_{W50} of 8.5 μM are markedly less sensitive to MT than *L. amazonensis* (c_{W50} values between 2.3 and 3.1 μM, Table 3). The c_w value for erythrocytes, which was determined from the 50% hemolysis data, was similar to the c_{w50} value for *Leishmania*. Clinical pharmacokinetic data indicated that the MT plasma concentration in visceral leishmaniasis patients peaks at ~90 μg/mL [26]. Hemolysis measurements in whole blood showed 2.8% hemolysis (not significant) for this plasma concentration and a significant level of 5.4% for a two-fold higher concentration [17]. Although the *Leishmania* c_{m50} values were essentially the same for the experiments with 2- or 24-h incubation periods, the c_{m50} was significantly higher for macrophages with a 24-h incubation period. In addition, in presence of FCS with a 24-h incubation period, the c_{w50} values for *Leishmania* and macrophages were approximately 5- and 15-fold higher, respectively. Collectively, these results suggest that this drug possesses a certain ability to penetrate macrophages. This finding is interesting and warrants further study because it could be related to the drug's efficacy in the treatment of leishmaniasis [8].

The cytotoxicity parameters presented here (c_{w50}, c_{m50} and K) allow a more detailed analysis of the drug's interaction with cell membranes and can be easily determined by collecting cytotoxicity data for various cell concentrations. However, the rationalization of these results based on the characteristics of each type of membrane is challenging. *Leishmania* promastigotes have a protective cell surface coat that mediates essential host-parasite interactions and is mainly composed of glycosylphosphatidylinositol (GPI)-anchored proteins and/or free GPI glycolipids [27]. The expression of the GPI-anchored proteins is downregulated in the intracellular amastigote, but the expression level of free GPI is maintained [28]. The macrophage membrane is characterized by the presence of Toll-like receptors (TLR), which play a key role in the innate immune response to invading pathogens [29]. TLRs are transmembrane glycoprotein receptors with extracellular, transmembrane and cytoplasmic domains. The erythrocyte membrane includes band 3 complex, glycophorins and Rh-complex as the principal transmembrane proteins and a membrane skeleton on the cytoplasmic side [30]. The lower content of outer peripheral proteins in erythrocyte membranes compared with the contents found in *Leishmania* and macrophage cells might explain why their lipid bilayers are more easily disrupted by MT.

The membrane-water partition coefficient (K) describes the affinity of a compound for biological membranes with respect to the laws of thermodynamics. A more practical parameter, the octanol–water partition coefficient (Log $P_{O/W}$), has been widely used as an estimate of Log K. In this study, we found that the membrane-culture medium partition coefficients for MT in *L. amazonensis* and macrophages were found to be more than three orders of magnitude higher than the reported Log $P_{O/W}$ value of 2.25 ($P_{O/W} = 178$) [31]. This discrepancy between the values of K and $P_{O/W}$ is another indication that MT can bind to biological membrane proteins in large quantities. In a previous study, the antiproliferative activities of the terpenes nerolidol, (+)-limonene, α-terpineol and 1,8-cineole against *L. amazonensis* promastigotes were assessed at various cell concentrations. Nerolidol was the most potent of the tested terpenes, exhibiting IC_{50} values ranging from 8 μM for 5×10^6 parasites/mL to 1.1 mM for 2×10^9 parasites/mL [32]. The K values for nerolidol (Log K = 5.33) and limonene (Log K = 4.23) were similar to their respective Log $P_{O/W}$ values. However, EPR spectroscopy of the spin label 6-MSL revealed that the terpenes, unlike MT, caused only small increases in the protein dynamics of the parasite membrane [32]. In contrast, the fatty acid spin label 5-DSA, which was used to monitor the lipid-protein interface in the membrane, indicated a marked increase in the fluidity of the terpene-treated *Leishmania* samples. Furthermore, the minimum terpene concentration required to change the membrane fluidity differed among the various terpenes, and the concentrations obtained were correlated with the corresponding IC_{50} values of the terpenes [32].

At its IC_{50}, MT has been demonstrated to induce programmed apoptosis-like cell death in promastigotes of *L. donovani* [33,34], *L. amazonensis* [35], *L. major* and *L. tropica* [36] as well as the unicellular parasite *Toxoplasma gondii* [37] and some yeast strains [6]. These experiments were generally performed using lower concentrations of cells (10^6–10^7 cells/mL) with incubation periods of 24–72 h in medium supplemented with FCS. The more dilute cell suspensions used in this study resulted in greater fractions of MT bound to the albumin in the FCS. Under these conditions, some of the MT is slowly released to the cells and could cause minor damage to the cell membrane, such as the formation of small pores accompanied by electrolyte leakage and consequent alteration of the membrane potential. Because any change in the plasma membrane potential is immediately reflected in the mitochondrial membrane potential, a certain fraction of cells in the suspension could trigger programmed apoptosis-like cell death. Our results indicated that MT at its IC_{50} produces a plasma membrane concentration of 1.8 M (Table 3), i.e., the estimated concentration in the membrane was 740,000-fold greater than that in aqueous media; therefore, its action on plasma membrane proteins is expected to be an important event. The experimental results of our present study revealed that MT does not simply pass through the plasma membrane to accumulate inside parasites but actually accumulates in their lipid and protein components at very high rates. Interestingly, the use of MT as a PI3K/Akt inhibitor has been reported to markedly reduce HIV-1 production from long-living virus-infected macrophages, and researchers have suggested that HIV-1 infection in macrophages induces the recruitment of Akt into the plasma membrane via its PH domain and that this effect could be reversed through the use of MT [38].

The experimental resistance of *Leishmania* to MT has been associated with decreased MT accumulation, which can be achieved by an increase in drug efflux mediated by overexpression of the ABC transporter P-glycoprotein or inactivation of any one of the two proteins responsible for MT uptake, namely the MT transporter LdMT and its beta subunit LdRos3 [39]. The experimental results obtained in the present study are consistent with an accumulation of MT in parasite membranes, particularly the plasma membrane, and not directly with its internalization. For the assay with FCS (24 h), the MT concentration accumulated in the membrane of amastigotes and promastigotes was estimated to be 144,000- and 68,000-fold greater than that in the aqueous medium, respectively (Table 3). We hypothesize that the LdMT-LdRos3-dependent flippase machinery potentiates the activity of MT against *Leishmania* parasites promoting a full distribution of the drug on the two leaflets of the plasma membrane [39]. In contrast, the overexpression of ABC transporters acting as MT floppases could promote a higher drug concentration in the outer leaflet of the plasma membrane. A similar result could be achieved in a cytotoxicity experiment using a short incubation period, which would be insufficient for complete distribution of MT in the membrane (Fig. 5). Based on this study, other *Leishmania* species and experimentally resistant parasites with higher IC_{50} values would be characterized by a lower membrane-culture medium partition coefficient and/or tolerance to a higher concentration of MT in the membrane (smaller K and/or greater c_{m50}, Table 3).

5. Conclusions

Experiments with different concentrations of cells in suspension resulted in significantly different IC_{50} values. These data were fitted by an equation that generated best-fit parameters, such as the drug concentrations in the solvent (c_{w50}) and in the cell membrane (c_{m50}) and the membrane-solvent partition coefficient (K). Interestingly, unlike the IC_{50} values, the three parameters discussed in this study are independent of the experimental cell concentration and provide new information regarding the cytotoxic effects of a drug. MT was less toxic to macrophages, with a c_{w50} value that was more than two-fold higher than that obtained for *Leishmania*. The estimated c_{m50} values for *Leishmania* amastigotes and macrophages were 1.8 and 2.5 M, respectively, after a 2-h incubation. For macrophages, the c_{m50} determined after a 24-h incubation period was significantly increased, suggesting that MT can enter macrophages and accumulate in their interiors. At its IC_{50}, MT increased the plasma membrane fluidity of *Leishmania* and macrophages, as shown by spin-labeling EPR spectroscopy. The membrane alterations observed at 0.7 mM MT using 2×10^9 cells/mL were also detected at a lower MT concentration (15 μM) using 4×10^7 *L. amazonensis* amastigotes/mL. These increases in membrane fluidity were accompanied by small amounts of cell lysis. We believe that the primary action of MT is on membrane proteins and might lead to electrolyte leakage and even membrane disruption in the range of IC_{50} concentrations.

Conflict of interest statement

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

This study was financially supported by grants from the Brazilian research funding agencies CNPq (445666/2014-5 and 302216/2010-3), CAPES and FAPEG (201210267001110). Kelly S. Fernandes was a recipient of a fellowship from CAPES. Antonio Alonso received a research grant from CNPq. The authors acknowledge Graziella G. Matos for the help provided in obtaining peritoneal macrophages.

References

[1] B. Monge-Maillo, R. López-Vélez, Miltefosine for visceral and cutaneous leishmaniasis: drug characteristics and evidence-based treatment recommendations, Clin. Infect. Dis. 60 (2015) 1398–1404, http://dx.doi.org/10.1093/cid/civ004.
[2] A.C. Aichelburg, J. Walochnik, O. Assadian, H. Prosch, A. Steuer, G. Perneczky, G.S. Visvesvara, H. Aspöck, N. Vetter, Successful treatment of disseminated *Acanthamoeba* sp. infection with miltefosine, Emerg. Infect. Dis. 14 (2008) 1743–1746, http://dx.doi.org/10.3201/eid1411.070854.
[3] W.J. Van Blitterswijk, M. Verheij, Anticancer alkylphospholipids: mechanisms of action, cellular sensitivity and resistance, and clinical prospects, Curr. Pharm. Des. 14 (2008) 2061–2074, http://dx.doi.org/10.2174/138161208785294636.
[4] D. Llull, L. Rivas, E. García, In vitro bactericidal activity of the antiprotozoal drug miltefosine against *Streptococcus pneumoniae* and other pathogenic streptococci, Antimicrob. Agents Chemother. 51 (2007) 1844–1848, http://dx.doi.org/10.1128/AAC.01428-06.
[5] F. Widmer, L.C. Wright, D. Obando, R. Handke, R. Ganendren, D.H. Ellis, T.C. Sorrell, Hexadecylphosphocholine (miltefosine) has broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis, Antimicrob. Agents Chemother. 50 (2006) 414–421, http://dx.doi.org/10.1128/AAC.50.2.414-421.2006.
[6] X. Zuo, J.T. Djordjevic, J. Bijosono Oei, D. Desmarini, S.D. Schibeci, K.A. Jolliffe, T.C. Sorrell, Miltefosine induces apoptosis-like cell death in yeast via Cox9p in cytochrome c oxidase, Mol. Pharmacol. 80 (2011) 476–485, http://dx.doi.org/10.1124/mol.111.072322.
[7] W.J. Van Blitterswijk, M. Verheij, Anticancer mechanisms and clinical application of alkylphospholipids, Biochim. Biophys. Acta 1831 (2013) 663–674, http://dx.doi.org/10.1016/j.bbalip.2012.10.008.
[8] T.P. Dorlo, M. Balasegaram, J.H. Beijnen, P.J. de Vries, Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis, J. Antimicrob. Chemother. 67 (2012) 2576–2597, http://dx.doi.org/10.1093/jac/dks275.
[9] L. Alonso, S.A. Mendanha, C.A. Marquezin, M. Berardi, A.S. Ito, A.U. Acuña, A. Alonso, Interaction of miltefosine with intercellular membranes of stratum corneum and biomimetic lipid vesicles, Int. J. Pharm. 434 (2012) 391–398, http://dx.doi.org/10.1016/j.ijpharm.2012.06.006.
[10] R.A. Moreira, S.A. Mendanha, D. Hansen, A. Alonso, Interaction of miltefosine with the lipid and protein components of the erythrocyte membrane, J. Pharm. Sci. 102 (2013) 1661–1669, http://dx.doi.org/10.1002/jps.23496.
[11] R.A. Moreira, S.A. Mendanha, K.S. Fernandes, G.G. Matos, L. Alonso, M.L. Dorta, A. Alonso, Miltefosine increases lipid and protein dynamics in *Leishmania amazonensis* membranes at concentrations similar to those needed for cytotoxicity activity, Antimicrob. Agents Chemother. 58 (2014) 3021–3028, http://dx.doi.org/10.1128/AAC.01332-13.
[12] M.C. Teixeira, R. de Jesus Santos, R.B. Sampaio, L. Pontes-de-Carvalho, W.L. dos-Santos, A simple and reproducible method to obtain large numbers of axenic amastigotes of different *Leishmania* species, Parasitol. Res. 88 (2002) 963–968, http://dx.doi.org/10.1007/s00436-002-0695-3.
[13] N. Gupta, N. Goyal, A.K. Rastogi, In vitro cultivation and characterization of axenic amastigotes of *Leishmania*, Trends Parasitol. 17 (2001) 150–153, http://dx.doi.org/10.1016/S1471-4922(00)01811-0.
[14] V.H. Hodgkinson, L. Soong, S.M. Duboise, D. McMahon-Pratt, *Leishmania amazonensis*: cultivation and characterization of axenic amastigote-like organisms, Exp. Parasitol. 83 (1996) 94–105, http://dx.doi.org/10.1006/expr.1996.0053.
[15] A. Ray, B.N. Dittel, Isolation of mouse peritoneal cavity cells, J. Vis. Exp. 35 (2010) 1488, http://dx.doi.org/10.3791/1488.
[16] M.W. Vaughn, K.T. Huang, L. Kuo, J.C. Liao, Erythrocyte consumption of nitric oxide: competition experiment and model analysis, Nitric Oxide 5 (2001) 18–31, http://dx.doi.org/10.1006/niox.2000.0328.
[17] L. Alonso, A. Alonso, Hemolytic potential of miltefosine is dependent on cell concentration: implications for in vitro cell cytotoxicity assays and pharmacokinetic data, Biochim. Biophys. Acta 1858 (2016) 1160–1164, http://dx.doi.org/10.1016/j.bbamem.2016.03.004.
[18] W.L. Hubbell, D.S. Cafiso, C. Altenbach, Identifying conformational changes with site-directed spin labeling, Nat. Struct. Biol. 7 (2000) 735–739, http://dx.doi.org/10.1038/78956.
[19] J.L. Anjos, P.S. Santiago, M. Tabak, A. Alonso, On the interaction of bovine serum albumin with ionic surfactants: temperature induced EPR changes of a maleimide nitroxide reflect local protein dynamics and probe solvent accessibility, Colloids Surf. B: Biointerfaces 88 (2011) 463–470, http://dx.doi.org/10.1016/j.colsurfb.2011.07.030.
[20] P.C. Jost, O.H. Griffith, R.A. Capaldi, G. Vanderkooi, Evidence for boundary lipid in membranes, Proc. Natl. Acad. Sci. U. S. A. 70 (1973) 480–484, http://dx.doi.org/10.1073/pnas.70.2.480.
[21] D. Marsh, Electron spin resonance in membrane research: protein-lipid interactions from challenging beginnings to state of the art, Eur. Biophys. J. 39 (2010) 513–525, http://dx.doi.org/10.1007/s00249-009-0512-3.
[22] M. Suleman, J.P. Gangneux, L. Legentil, S. Belaz, Y. Cabezas, C. Manuel, R. Dureau, O. Sergent, A. Burel, F. Daligault, V. Ferrières, F. Robert-Gangneux, Alkyl galactofuranosides strongly interact with *Leishmania donovani* membrane and provide antileishmanial activity, Antimicrob. Agents Chemother. 58 (2014) 2156–2166, http://dx.doi.org/10.1128/AAC.01350-13.
[23] A. Alonso, C.S. Queiroz, A.C. Magalhães, Chilling stress leads to increased cell membrane rigidity in roots of coffee (*Coffea arabica* L.) seedlings, Biochim. Biophys. Acta 1323 (1997) 75–84, http://dx.doi.org/10.1016/S0005-2736(96)00177-0.
[24] M.F. Nepomuceno, A. Alonso, L. Pereira-da-Silva, M. Tabak, Inhibitory effect of dipyridamole and its derivatives on lipid peroxidation in mitochondria, Free Radic. Biol. Med. 23 (1997) 1046–1054, http://dx.doi.org/10.1016/S0891-5849(97)00135-4.
[25] S.L. Croft, K. Seifert, M. Duchêne, Antiprotozoal activities of phospholipid analogues, Mol. Biochem. Parasitol. 126 (2003) 165–172, http://dx.doi.org/10.1016/S0166-6851(02)00283-9.
[26] T.P. Dorlo, A.D. Huitema, J.H. Beijnen, P.J. de Vries, Optimal dosing of miltefosine in children and adults with visceral leishmaniasis, Antimicrob. Agents Chemother. 56 (2012) 3864–3872, http://dx.doi.org/10.1128/AAC.00292-12.
[27] P.F. Pimenta, E.M. Saraiva, D.L. Sacks, The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*, Exp. Parasitol. 72 (1991) 191–204, http://dx.doi.org/10.1016/0014-4894(91)90137-L.
[28] S.C. Ilgoutz, M.J. McConville, Function and assembly of the *Leishmania* surface coat, Int. J. Parasitol. 31 (2001) 899–908, http://dx.doi.org/10.1016/S0020-7519(01)00197-7.
[29] T. Kawai, S. Akira, TLR signaling, Semin. Immunol. 19 (2007) 24–32, http://dx.doi.org/10.1016/j.smim.2006.12.004.
[30] S. Perrotta, P.G. Gallagher, N. Mohandas, Hereditary spherocytosis, Lancet 372 (2008) 1411–1426, http://dx.doi.org/10.1016/S0140-6736(08)61588-3.
[31] Chemicalize.org, ChemAxon, http://www.chemicalize.org/structure/#!mol=Miltefosine 2016 (accessed 30.05.16).
[32] H.S. Camargos, R.A. Moreira, S.A. Mendanha, K.S. Fernandes, M.L. Dorta, A. Alonso, Terpenes increase the lipid dynamics in the *Leishmania* plasma membrane at concentrations similar to their IC_{50} values, PLoS One 9 (2014), e104429, http://dx.doi.org/10.1371/journal.pone.0104429.
[33] C. Paris, P.M. Loiseau, C. Bories, J. Bréard, Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes, Antimicrob. Agents Chemother. 48 (2004) 852–859, http://dx.doi.org/10.1128/AAC.48.3.852-859.2004.

[34] N.K. Verma, G. Singh, C.S. Dey, Miltefosine induces apoptosis in arsenite-resistant *Leishmania donovani* promastigotes through mitochondrial dysfunction, Exp. Parasitol. 116 (2007) 1–13, http://dx.doi.org/10.1016/j.exppara.2006.10.007.

[35] F. de Aquino Marinho, K.C. da Silva Gonçalves, S. Soares de Oliveira, A.C. de Siqueira Couto de Olivia, M. Bellio, C.M. d'Avila-Levy, A.L.S.d. Santos, M.H. Branquinha, Miltefosine induces programmed cell death in *Leishmania amazonensis* promastigotes, Mem. Inst. Oswaldo Cruz 106 (2011) 507–509.

[36] S. Khademvatan, M.J. Gharavi, F. Rahim, J. Saki, Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains, Korean J. Parasitol. 49 (2011) 17–23, http://dx.doi.org/10.3347/kjp.2011.49.1.17.

[37] A.D. Ni Nyoman, C.G. Lüder, Apoptosis-like cell death pathways in the unicellular parasite *Toxoplasma gondii* following treatment with apoptosis inducers and chemotherapeutic agents: a proof-of-concept study, Apoptosis 18 (2013) 664–680, http://dx.doi.org/10.1007/s10495-013-0832-8.

[38] P. Chugh, B. Bradel-Tretheway, C.M. Monteiro-Filho, V. Planelles, S.B. Maggirwar, S. Dewhurst, B. Kim, Akt inhibitors as an HIV-1 infected macrophage-specific anti-viral therapy, Retrovirology 5 (2008) 11, http://dx.doi.org/10.1186/1742-4690-5-11.

[39] F.J. Pérez-Victoria, M.P. Sánchez-Cañete, K. Seifert, S.L. Croft, S. Sundar, S. Castanys, F. Gamarro, Mechanisms of experimental resistance of leishmania to miltefosine: implications for clinical use, Drug Resist. Updat. 9 (2006) 26–39, http://dx.doi.org/10.1016/j.drup.2006.04.001.