Membrane sterol depletion impairs miltefosine action in wild-type and miltefosine-resistant *Leishmania donovani* promastigotes

M. Saint-Pierre-Chazalet¹–⁴, M. Ben Brahim¹,², L. Le Moyec⁴,⁵, C. Bories¹,², M. Rakotomanga¹,² and P. M. Loiseau¹,²*

¹Université Paris-Sud, UMR 8076, Chimiothérapie Antiparasitaire, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, Chatenay-Malabry, F-92296, France; ²CNRS, Chatenay-Malabry, F-92296, France; ³UPMC Université Paris 06, BioMoCeTi, Genopole Campus 1, 5 rue Henri Desbruères, 91030 Evry cedex, France; ⁴CNRS UMR 7033, BioMoCeTi, Genopole Campus 1, 5 rue Henri Desbruères, 91030 Evry cedex, France; ⁵Université Paris 13, UFR SMBH, 74 rue Marcel Cachin, 93017 Bobigny, France

Received 1 April 2009; returned 3 June 2009; revised 24 July 2009; accepted 3 August 2009

**Objectives**: This study focuses on the importance of sterols in the action of miltefosine (hexadecylphosphocholine, HePC) against *Leishmania donovani*.

**Methods**: Plasma membranes of *L. donovani* promastigotes were depleted of sterol using methyl-β-cyclodextrin (MCD) and cholesterol oxidase (CH-OX). Sterols were quantified and HePC susceptibility was assessed using the MTT test. A biomimetic model of the outer leaflet of a *Leishmania* plasma membrane was used to decipher the HePC–lipid interactions.

**Results**: CH-OX, which is known to act more specifically on condensed membranes, therefore at the level of lipid rafts, gave a better extraction yield in HePC-resistant parasites, confirming the more rigid structure of their membranes than those of wild-type parasites. Sterol depletion was responsible for a 40% decrease in HePC susceptibility in both wild-type and HePC-resistant parasites. Sterol repletion of the sterol-depleted parasites restored HePC susceptibility. The biomimetic model of the outer leaflet of a *Leishmania* plasma membrane confirmed that condensed microdomains were able to incorporate higher quantities of HePC than fluid ones and this result was amplified when the sterol concentration was increased.

**Conclusions**: Sterol and lipid rafts probably play a significant role as an HePC reservoir providing a constant supply to the previously described transporter. In addition, ¹H NMR experiments suggested that HePC stimulated lipid trafficking in parasites.

Keywords: antileishmanial action, chemotherapy, drug resistance, mechanism of action

**Introduction**

Leishmaniases are a family of tropical and subtropical parasitic diseases provoked by Euglenozoa parasites of the genus *Leishmania*. Visceral leishmaniasis (VL) is the most severe form because of its lethality if not treated (http://www.who.int/leishmaniasis/en/). Among the various species able to infect humans, *Leishmania donovani* is the causative species on the Indian subcontinent and in eastern Africa, affecting 62 countries, with a total of 200 million people at risk.¹ The annual incidence is estimated at ~500 000 cases.² At the moment, chemotherapy remains the most efficient way to control leishmaniasis despite efforts developed both to control the insect vector and to find a vaccine.³ Miltefosine (hexadecylphosphocholine, HePC) is an alkylphosphocholine that was first developed as an anticancer drug, particularly active against breast cancer metastases⁴ (Figure 1). Moreover, HePC has antileishmanial properties and was registered as the first orally active antileishmanial drug under the tradename of Impavido® for treatment of VL in India in 2002 and in Germany in 2004 and also for treatment of...
cutaneous leishmaniasis in Colombiamain2005. The mechanism of HePC action has not been completely elucidated but the available data suggest that several targets might be affected within the parasite. HePC enters the parasite via a P-type ATPase transporter called LdMT. We previously described that HePC was responsible for an apoptosis-like death in L. donovani promastigotes and similar results were found in amastigotes.

We observed HePC interference with phospholipid metabolism that is differentially expressed in HePC-susceptible and HePC-resistant L. donovani. HePC was also found to inhibit cytochrome C oxidase, and this action could be related to the apoptosis induced by HePC. These findings suggested that HePC internalization has various consequences on cell signalling. The present article is focused on drug–membrane interactions, since we found a condensation effect of HePC suggesting a strong affinity for sterols, using a lipid monolayer model that mimicked the external monolayer of a plasma membrane from L. donovani promastigotes. This affinity of HePC for sterols suggests that lipid rafts, previously described in Leishmania by Denny et al., could be a reservoir of HePC at the level of the L. donovani plasma membrane. In order to verify this assumption, we monitored the HePC susceptibility of parasites that had been depleted of sterols.

Materials and methods

Chemical compounds

HePC was kindly supplied by Zentaris (Frankfurt, Germany). Methyl-β-cyclodextrin (MCD) was supplied by Wacker Laboratories (Burghausen, Germany). Cholesterol oxidase (CH-OX) from Escherichia coli was purchased from Sigma (Saint-Quentin Fallavier, France). 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylethanolamine (POPE), 1,2-diesteroyl-sn-glycerol-3-phosphatidylcholine (DSPC), 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylglycerol (DPPG) and ergosterol (99% pure) were also purchased from Sigma. The lipids were dissolved in a chloroform/ethanol (4:1, v/v) mixture at a concentration of 10 M.

Parasite strains and culture

Promastigotes forms of wild-type (WT) and HePC-resistant (HePC-R) L. donovani LV9 (MHOM/ET/67/HU3) lines were grown in M-199 medium (Sigma) supplemented with 10% inactivated fetal calf serum (Invitrogen, Eragny, France), 40 mM HEPES (VWR, Paisley, Scotland), 100 μM adenosine (Sigma) and 0.5 mg/L haemin (Sigma) in the presence of 50 mg/L gentamicin at 26°C in a dark atmosphere. The HePC-R line was cultivated in the presence of 40 μM HePC.

Sterol depletion

For sterol depletion, L. donovani promastigotes were cultured in flasks at an initial density of 10⁶ promastigotes/mL in 100 mL of the medium described above. The flasks were placed in an orbital incubator under continuous shaking (150 rpm) at 26°C. At the end of the logarithmic phase, WT and HePC-R promastigotes (∼10⁶ parasites/mL) were harvested by centrifugation (4000 g, 4°C, 10 min) and washed three times with large volumes of serum-free medium (SFM) to eliminate all traces of sterol in the medium that would reduce the efficiency of sterol depletion. The pellet was then suspended in 6 mL of SFM and this suspension was then divided into two equal parts: one control sample and one sample incubated with MCD or CH-OX for 2 h at 26°C. After this period of sterol depletion, parasites were washed in PBS pH 7.2 to eliminate MCD–sterol complexes or CH-OX. The pellet was suspended in SFM and the cell density was adjusted to 4×10⁸ parasites/mL prior to determining their drug susceptibility.

Sterol repletion

Cholesterol was made available to the sterol-depleted parasites from a soluble complex of cholesterol–MCD. MCD (458.5 mg) was dissolved in 2 mL of PBS pH 7.2. Cholesterol (0.6 g) was then added, giving a solubilized cholesterol concentration of 100 mM and an MCD concentration of 70 mM. The mixture was stirred overnight at room temperature and then filtered through a 0.45 μm filter to recover the soluble complex. The sterol-depleted parasite suspension at 4×10⁸ parasites/mL was incubated for 24 h period with the soluble complex to obtain a final cholesterol concentration of 10 mM. After three washings with SFM to eliminate MCD, the susceptibility to HePC was determined as described below.

Determination of cholesterol content

Depleted parasites and controls were washed in 1× PBS (Sigma). Then they were centrifuged (4000 g, 4°C, 10 min) and the pellet was lysed in 50 mM Tris-HCl, 5 mM EDTA pH 7.4 buffer to disrupt the parasites. Parasite disruption was complete after the suspension was sonified by six cycles of 30 s each at 0°C. Sterols were assayed using the colorimetric cholesterol quantification kit (ClniSciences, Montrouge, France) based on the cholesterol esterase/CH-OX combined system and performed on a C6000 Roche/Hitachi analyser (Paris, France). We checked the specificity of the assay using pure compounds and found that this system quantified all sterols, not only cholesterol but also ergosterol, present in L. donovani.

Assessment of parasite integrity after sterol depletion

The conditions of sterol depletion were optimized after incubation of parasites in the presence of MCD (5, 10, 20 and 40 mM) or CH-OX (1 and 2 U/mL) for 30 min, 1 h and 2 h. Parasite morphology and motility were observed using an optical microscope and parasite viability was assessed using the MTT test described below. The reversibility of the sterol depletion effects was evaluated as follows: after sterol depletion and three successive washings to eliminate the depletion agents, parasites were subcultured for a period of 7 days and the viability of the subculture was determined microscopically.

HePC susceptibility

HePC susceptibility was determined in flat-bottomed 96-well plastic tissue-culture plates maintained at 26°C in an atmosphere of 5% CO₂. Each well of the plates was filled with 100 μL of the sterol-depleted or control parasite suspension adjusted to 4×10⁸ parasites/mL,
Sterol depletion impairs antileishmanial action of miltefosine

and plates were incubated at 27°C for 1 h before drug addition. HePC solutions were added so as to attain the final desired concentrations (200, 100 and 20 µM) with each concentration in triplicate. After a 1 h incubation period at 27°C in the dark and under a 5% CO2 atmosphere, the viability of promastigotes was assessed using the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or MTT, colorimetric method, which measures the reduction of this tetrazolium compound into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent, a detergent solution (Triton X-100, HCl) was added to lyse the cells and dissolve the coloured crystals. The absorbance at 570 nm, directly proportional to the number of viable cells, was measured using an ELISA plate reader. The results are expressed as the residual mitochondrial activity as a percentage of that of untreated control parasites after a 1 h incubation.

Lipid monolayer study

Lipid monolayers were prepared as previously described by using a Teflon trough provided by Riegler (Riegler and Kirstein GmbH Wiesbaden, Germany). The trough (6.2×26.3×0.5 cm) was filled with Millipore water (pH 5.6). A solution of lipid in chloroform/ethanol (4:1, v/v) was spread at the air/water interface. The surface pressure was measured by the Wilhelmy method, by means of a thin plate of filter paper. An electronic device enabled us to keep the surface pressure constant by monitoring the displacement of the barriers. All experiments were performed at 21±1°C. The speed of compression and decompression of the barriers (3×10−2 cm s−1) was kept constant during the experiments. Phospholipids and ergosterol are amphiphilic molecules which, spread at the air/water interface, form a homogeneous stable monolayer. After compression of the monolayer, we obtained isotherms (pressure/area per molecule). Exogenous molecules were then injected into the subphase below the monolayer to study the interactions of these molecules with the lipid monolayer. In order to study the interaction of HePC with lipid monolayers, the monolayer of lipid was compressed to 25 mN/m. Generally, the surface pressure of a biological membrane is estimated at 30 mN/m, but in this case 25 mN/m was used to obtain reliable measurements, because 30 mN/m is too close to the collapse pressure of HePC. The surface pressure (P) was kept constant (25 mN/m) and an aqueous solution of HePC was injected with a microsyringe under the monolayer at final concentrations in the range 0.2–4 µM, according to the process described previously. If an interaction occurred between the molecules of the subphase and the monolayer, the barriers moved back to keep the pressure to 25 mN/m and the variation of the mean molecular area ΔA of lipid monolayer versus time was recorded for 60 min (adsorption kinetics). After 1 h of adsorption, a new isotherm (lipid with HePC) was recorded.

The maximum percentage of HePC monomers (%HePCmax) inserted into the lipid monolayer is calculated from the following formula:\(^ {16}\)

\[ \% \text{HePC}_{\text{max}} = \left( \Delta A_{\text{max}} / A_{\text{HePC}} \right) / \left( 1 + \Delta A_{\text{max}} / A_{\text{HePC}} \right) \]

where ΔA_{max} is the maximum variation of the area per molecule of lipid at the constant surface pressure and A_{HePC} is the cross-sectional area of HePC. From a previous paper, HePC compression isotherms yielded A_{HePC}=26 Å² (see de Certaines et al.\(^ {16}\)).

NMR proton spectroscopy

L. donovani promastigotes (WT or treated with 20 µM or 40 µM HePC for a 48 h period) were collected and washed once with phosphate buffer and three times with phosphate buffer in D2O. The final pellet containing ~10⁷ cells was placed with phosphate buffer in a Shigemi NMR tube in a final volume of 350 µL. Proton spectra were acquired at 400 MHz on a Bruker Advance spectrometer at 300 K. The 1D acquisition sequence included the presaturation of the residual water signal. The spectral width was 4800 Hz acquired on 16 K data points. Each 90° pulse was followed by a 5 s relaxation delay that included the presaturation pulse. The FID consisted of 64 acquisitions. The spectra were processed with WINNMR software to measure the area of each resonance. The FID was Fourier transformed after an exponential filter of 1 Hz line broadening. The areas were measured and reported to that of methyl resonance at 0.8 ppm. The experiments were repeated seven times for WT parasites and four times for parasites treated with 20 µM or 40 µM HePC.

Statistical analysis

The statistical significance of mitochondrial activity versus HePC concentration was analysed using a Kruskal–Wallis test and a t-test was used to compare the viability of the strain treated or not treated with MCD or CH-OX at each HePC concentration. The statistical significance was tested with a threshold of P<0.05.

Results

Lipid rafts are assumed to be microdomains particularly rich in sterols. Such microdomains have been described in Leishmania spp. by Denny et al.\(^ {12}\) Considering the affinity of HePC for sterols that we described previously,\(^ {11}\) we hypothesized that HePC could accumulate in these sterol-enriched microdomains and therefore lipid rafts could be involved in the action of HePC in L. donovani. One strategy to test this hypothesis was to destabilize lipid rafts and then to measure the susceptibility of the parasites to HePC.

Sterol depletion in L. donovani plasma membranes

We therefore attempted sterol depletion of the parasite plasma membrane by two methods, one using MCD and the other using CH-OX. The conditions of sterol depletion were optimized to preserve morphology, motility and viability by varying two parameters: the concentration of the depletion agent and the time of extraction. For MCD, the best conditions allowing total reversibility of the effects on culture growth after sterol depletion consisted of incubation of the parasites with 10 mM MCD for a 2 h period (Table 1). After sterol depletion cell viability as assessed by mitochondrial activity was decreased by 23% in WT and by 20%–22% in HePC-R parasites (data not shown); therefore, sterol depletion did not have a significantly different impact on WT and HePC-R parasite viability. Sterol depletion using CH-OX was also optimized to the conditions of incubating 1 U of CH-OX/mL with 10⁸ parasites. In contrast to MCD, CH-OX did not have any effect on parasite morphology and motility. The sterol depletion by CH-OX caused a slight decrease in mitochondrial activity (2% in WT and 10% in HePC-R parasites). We then quantified the amount of sterol remaining after depletion. Figure 2 shows that the initial sterol content before depletion in HePC-R parasites was about twice as high as that in WT. When using MCD for sterol depletion, the sterol extraction was more efficient in WT (28% reduction in the sterol content)
than in HePC-R (13%). On the other hand, the use of CH-OX was responsible for more extensive sterol depletion in HePC-R (33%) than in WT (6%). These sterol-depleted parasites were then incubated with HePC for a 1 h period to evaluate the effect of sterol depletion on the susceptibility to the drug. Sterol depletion using MCD significantly decreased the susceptibility of WT to HePC (Figure 3a) whereas the use of CH-OX did not yield a significant difference although a slight decrease in HePC susceptibility could be noted (Figure 3b). These results are positively correlated with the sterol extraction yield obtained with MCD and CH-OX in WT parasites (Figure 2). In contrast, the sterol depletion in HePC-R parasites using CH-OX led to a larger decrease in HePC susceptibility than when MCD was used (Figure 4a and b). These results are also correlated with the extent of sterol extraction (Figure 2).

Sterol repletion
Sterol repletion of the sterol-depleted parasites allowed the parasites to recover their initial susceptibility to HePC (data not shown). Such a reversibility in HePC susceptibility as a function of sterol exposure confirms the importance of the amount of sterol in the HePC susceptibility of \(L.\) donovani promastigotes.

Interaction between HePC and the biomimetic monolayer
We studied the behaviour of HePC on a monolayer that was biomimetic of the outer leaflet of the promastigote plasma membrane using the Langmuir method. The choice of the components of this monolayer was determined from the results obtained in previous lipidomic studies performed on \(L.\) donovani WT plasma membranes.\(^{17}\) The main acyl chains of phospholipids were octadecanoic acid C18:0, 9-octadecenoic C18:1 and hexadecanoic C16:0. Ergosterol is one of the major \(Leishmania\) sterols.\(^{17}\) The major polar lipids of \(Leishmania\) membranes were reported to be mainly phosphatidylcholine (PC), phosphatidyl-lethanolamine (PE) and anionic phospholipids.\(^{17}\) Taking into account these results, we reconstituted a biomimetic monolayer composed of the main phospholipids and sterols identified with
Sterol depletion impairs antileishmanial action of miltefosine

The isotherm of the biomimetic monolayer reported in Figure 5 shows that the monolayer is in a liquid extended phase, but less fluid than a pure POPE monolayer. Adsorption kinetics of different concentrations of HePC injected under the biomimetic monolayer at 25 mN/m were recorded over 1 h (results not shown). The variations of the mean molecular area (ΔA) of the lipid monolayer observed 1 h after injecting HePC under the monolayer as a function of HePC concentration are reported in Figure 6. As previously described with pure fluid phospholipid monolayers (POPE and POPC),9,11 HePC monomers progressively inserted into the biomimetic monolayer up to a concentration of 2.5 μM corresponding to the critical micellar concentration (CMC).11 Above the CMC, insertion of HePC molecules occurred by both adsorption of independent monomers and deployment of HePC micelles as groups of monomers into the monolayer. This result was confirmed by the behaviour of the compression isotherms (Figure 7) recorded 1 h after injection of HePC under a monolayer at 25 mN/m. At high pressure, when the concentration of HePC was below the CMC, HePC monomers remained within the membrane. The presence of a plateau at 37 mN/m obtained for a concentration of HePC above the CMC was due to the ejection of groups of HePC monomers in the form of micelles.

Figure 3. Determination of the susceptibility to HePC of L. donovani WT promastigotes after sterol depletion using MCD (n = 3) and CH-OX (n = 3). The sterol depletion consisted of pre-incubation of parasites (10⁸ parasites/mL) with 10 mM MCD or 1 U/mL CH-OX for a 2 h period. After washing, parasites were incubated with HePC for a 1 h period to assess their susceptibility to HePC. The OD values for 100% mitochondrial activity ranged from 0.800 to 0.950. (a) WT with MCD versus WT without MCD at 20, 100 and 200 μM, significant (P < 0.05). (b) WT with CH-OX versus WT without CH-OX at 20, 100 and 200 μM, not significant.

Figure 4. Determination of the susceptibility to HePC of L. donovani HePC-R promastigotes after sterol depletion using MCD (n = 3) and CH-OX (n = 3). The sterol depletion consisted of pre-incubation of parasites (10⁸ parasites/mL) with 10 mM MCD or 1 U/mL CH-OX for a 2 h period. After washing, parasites were incubated with HePC for a 1 h period to assess their susceptibility to HePC. The OD values for 100% mitochondrial activity ranged from 0.800 to 0.950. (a) HePC-R with MCD versus HePC-R without MCD at 20 and 100 μM, not significant; HePC-R with MCD versus HePC-R without MCD at 200 μM, significant (P < 0.01). (b) HePC-R with CH-OX versus HePC-R without CH-OX at 20 μM, not significant; HePC-R with CH-OX versus HePC-R without CH-OX at 100 and 200 μM, significant (P < 0.01).

Role of sterols in HePC insertion in the biomimetic monolayer

The behaviour of HePC in the presence of pure fluid phospholipid monolayers (POPE and POPC), pure condensed phospholipid monolayers (DPPC, DPPE and DPPG) and a pure ergosterol monolayer has been previously described.9,11 From these results, in Table 2, we have reported the average ΔAmax variation obtained after monomer insertion at an HePC concentration of 2 μM into fluid and condensed phospholipid monolayers in comparison with the results obtained with a biomimetic monolayer. The results show that at the ΔAmax of 25 ± 2 Å²/molecule, the calculated %HePCmax values corresponding to the maximum yield of insertion of HePC monomers were higher in the biomimetic monolayer (50%) than in pure fluid or condensed phospholipid monolayers (37% and 28%, respectively) (Table 2). Since the fluidity of a biomimetic monolayer is intermediate between POPE (fluid monolayer) and DSPC (condensed monolayer) (Figure 7), the %HePCmax would be expected to be in the range 28%–37%, whereas it was found to be 50% (Table 2). The presence of 30% ergosterol in the biomimetic monolayer could explain this result, with the sterol enhancing HePC adsorption.
Lipid trafficking

As HePC is a lipid-like molecule, we verified whether HePC was able to stimulate lipid trafficking from internal compartments to the plasma membrane. The proton NMR spectra obtained on whole promastigote parasites are presented in Figure 8. As the spectral region beyond 6 ppm did not show any peaks, the region between 0 and 6 ppm is presented here. As in many other cell types, including cancer cells, the spectra were dominated by the signal arising from lipid molecules. In NMR spectra, the intensity of a signal (here the peak surface) depends not only on the amount of the molecules present in the sample but also on their mobility. For this reason, the peaks corresponding to lipids detected in whole cells are usually called ‘mobile’ lipids and have been shown to belong to a pool of lipids with low organization such as lipid droplets rather than to highly organized lipids in the membrane bilayer. These mobile lipids are likely to participate in cellular lipid trafficking. In the Leishmania parasites, such mobile lipids were detected and they were susceptible to HePC treatment, as shown in Figure 8. The results of the signal area measurements are given in Figure 9. They confirmed the appearance of the spectra. When normalized to the methyl function peak area, the methylene functions were increased dose dependently by HePC. With the highest dose of HePC, some significant differences could be found: the ratios to methyl function of methylene in the β position of the ester function (ratio C/A) or methylene beside ethyl function (D/A) or between ethyl function (F/A). When peaks C–H were compared with the surface of peak B, no statistical differences could be found.

Discussion

The description of lipid rafts in L. donovani promastigotes has opened up a new field of investigation into the mechanism of action of antileishmanial drugs. Drug–membrane interaction is a prerequisite for drug uptake before the active molecule reaches its intracellular targets. Some years ago, we reported that HePC has a strong affinity for membrane sterol using the Langmuir monolayer model. Taking into account the presence of lipid rafts in L. donovani promastigotes and the high level of sterols in these microdomains, we hypothesized that HePC could have a particular...
Affinity for lipid rafts in *L. donovani* promastigotes. In addition, the *Leishmania* membranes also contain ergosterol, described as more efficient than cholesterol at forming lipid rafts. First, the optimal conditions of sterol depletion were determined, ensuring reversible effects that maintained cell integrity and viability. Among the two methods used (MCD and CH-OX), MCD provoked a greater alteration of morphology, motility and cell viability. However, it should be noted that MCD has less specificity in sterol extraction than CH-OX, since MCD can also extract phospholipids and proteins leading to extensive modifications of the cytoskeleton, as observed microscopically in our study. It has previously been shown that prolonged treatment with MCD provoked sterol extraction in regions other than lipid rafts. The method using CH-OX is more specific than those using MCD since CH-OX binds preferentially to sterols of condensed and ordered domains. CH-OX catalyses specific oxidation of raft cholesterol to cholestenone, which is ejected from lipid rafts because of a modification of its polarity that modifies its interaction with surrounding phospholipids. In WT parasites, the most efficient sterol depletion was obtained with MCD, whereas in HePC-R parasites the most efficient sterol depletion was obtained with CH-OX. This difference could be ascribed to the different membrane compositions of WT and HePC-R lines. The membranes of HePC-R parasites are more rigid than those of WT.

**Table 2.** Average of maximum $\Delta A$ variation ($\Delta A_{\text{max}}$) of pure fluid phospholipids (POPE or POPC), pure condensed phospholipids (DPPE, DPPG, or DPPC) and biomimetic monolayer, after adsorption of monomers of HePC ($C_{\text{HePC}}=2\, \mu\text{M}$) at a constant surface pressure of 25 mN/m, temperature $21 \pm 1^\circ\text{C}$, pH=5.6

<table>
<thead>
<tr>
<th>Lipids</th>
<th>$\Delta A_{\text{max}} \pm 2, \text{Å}^2$ molecule at $2, \mu\text{M} , C_{\text{HePC}}$</th>
<th>$% C_{\text{HePC}}_{\text{max}}$ monomers with respect to total lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid phospholipids</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td>Condensed phospholipids</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Biomimetic membrane</td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

$\% C_{\text{HePC}}_{\text{max}}$, maximum percentage of HePC monomers inserted into the lipid monolayer.

**Figure 8.** 400 MHz NMR proton spectra of HePC-treated cells. The region between 0.5 and 6.5 ppm containing the resonance from mobile lipids is presented. A–F and H peaks arise from the fatty acid functions and the G peak arises from the $N$-trimethyl group of choline. A, $\text{CH}_2$; B, $\text{CH}_2$; C, $\text{CH}_2\text{CH}_2\text{C}=\text{O}$; D, $\text{CH}_2\text{CH}=\text{CH}$; E, $\text{CH}_2\text{C}=\text{O}$; F, $\text{CH}═\text{CH}\text{CH}_2\text{CH}=\text{CH}$; H, $\text{CH}=\text{CH}$.

**Figure 9.** Variation of motile lipid ratios: quantitative NMR analysis of lipid peaks A–H as shown in Figure 8, was performed in WT parasites and parasites treated with 20 and 40 $\mu\text{M}$ HePC. Statistical analysis was performed by ANOVA followed by a Student–Neumann–Keuls test and $P<0.05$ was considered as statistically different. Asterisks represent the treated groups significantly different from the WT group.
parasites because of the presence of more saturated phospholipid acyl chains. This membrane rigidity renders sterol extraction by MCD more difficult, resulting in lower sterol depletion in the HePC-R parasites. These results show therefore that sterol extraction by MCD was more efficient in fluid membranes and sterol extraction using CH-OX was more efficient in condensed membranes. Since lipid rafts are condensed microdomains, sterol extraction using CH-OX will act mainly at the level of lipid rafts.

HePC susceptibility was assessed using a sterol-free medium and under these conditions, the determination of HePC activity after a 72 h incubation period following a classical protocol was not possible because the cultures cannot grow without cholesterol in the medium. Cholesterol is not biosynthesized in Leishmania parasites, which have to take it up from the external medium. In order to overcome this limitation, it was necessary to choose drastic conditions to detect any differences in HePC susceptibility by using a high cell density, high HePC concentrations and a short incubation time. Under these conditions, we clearly demonstrate that HePC susceptibility was diminished when the sterol content decreased in the parasites, confirming the role of sterols in the action of HePC, as we previously described using a theoretical biomimetic model by the Langmuir method. In addition, sterol repletion of sterol-depleted parasites restored their initial susceptibility to HePC.

The biomimetic study using the Langmuir monolayer showed that at a surface pressure of 30 mN/m, corresponding to that of a biological membrane, HePC remained inserted within the monolayer as monomers. When the HePC concentration was higher than the CMC, the groups of monomers derived from micelles were highly concentrated in the monolayer and a part of these monomers could be ejected. Once HePC is inserted within the outer leaflet of the membrane, the flippase previously described can internalize it. Moreover, the biomimetic monolayer contains a mixture of lipids: fluid phospholipids such as POPE; rigid phospholipids such as DSPC and DPPG; and ergosterol. When these lipids are spread at the air/water interface, their distribution is not homogeneous and the measured surface pressure is that of the mean cohesion of the monolayer. The biomimetic monolayer was fluid overall, but some domains could be more condensed as in a cell membrane. On the other hand, when the monolayer was composed of a single fluid lipid (POPC or POPE), it was homogeneous. Thus, HePC monomers inserted more easily into the biomimetic monolayer than into a fluid phospholipid monolayer and this phenomenon could be ascribed to the presence of condensed domains induced by sterols. Such domains in the biomimetic monolayers could be visualized in further experiments using Brewster Angle Microscopy (BAM). This result suggests a better insertion of HePC into sterol-containing microdomains as a result of the high affinity of HePC towards sterols described previously. In the first monolayer experiments, we studied the influence of glycoinositolphospholipids (GIPLs), which are known to be present within Leishmania membranes, and we found that GIPLs had the same behaviour as DPPG. Since the behaviour of these two molecules appeared to be similar, we continued the studies with DPPG, which is more accessible.

In addition, we have treated L. donovani promastigotes with HePC and ketoconazole, an inhibitor of ergosterol biosynthesis, to observe whether a decrease in the ergosterol content had some influence on the action of HePC. We used the isobologram construction to detect the kind of interaction (antagonistic, additive or synergistic) between the two drugs. We found only an additive effect (data not shown). In a previous study, we determined the sterol composition in ketoconazole-treated L. donovani promastigotes and we found a collapse of ergosterol content and an accumulation of two other sterols as ergosterol precursors. However, the global sterol amount was maintained in ketoconazole-treated parasites. As we have previously demonstrated that HePC had similar affinity for ergosterol, cholesterol and other sterols, it is not surprising that the in vitro HePC action on Leishmania promastigotes was not modified by ketoconazole.

The treatment of the WT parasites with HePC induced modifications of their NMR spectra suggesting changes in lipid mobility. Two phenomena may increase the A/B peak ratio: a change in fatty acid chain length; or increased mobility of the methylene function in the lipids. The second is more likely to be happening here, because the other peak areas to methylene peak area ratios were not changed, and because the C/A ratio was also modified. This last ratio had no reason to change when the acyl chain length is increased, but may be affected by a better mobility of lipids. In the same manner, the degree of unsaturation in the acyl chains appeared to be unchanged (H/A ratio constant) but the mobility of the neighbouring peaks (F and D) may be affected with the HePC highest dose. Consequently, increased mobility seems to be responsible for the increased intensity of mobile lipid signals. This analysis is confirmed by previous lipidomic experiments after HePC treatment.

The presence of cholesterol within the membrane was reported to influence the membrane lipid fluidity. We have shown in cancer cells that modification of cholesterol content (by MCD) and the presence of raft microdomains increased mobile lipids. Here, in Leishmania parasites, we found that HePC has a similar effect to MCD on the mobile lipid NMR signal. The increased mobility is therefore in favour of an action of HePC on the parasite sterol content.

In conclusion, this study demonstrates that membrane sterols and lipid raft integrity are involved in the antileishmanial action of HePC since its activity is reduced by ~40% when the parasite membranes have reduced sterol content. The role of sterols and lipid rafts as HePC reservoirs at the membrane level probably contributes to making HePC available for interaction with the LdMT transporter, responsible for its internalization within the parasite. Further studies will be undertaken by purifying lipid rafts from L. donovani promastigotes and assaying HePC in these enriched fractions by comparison with the HePC content in non-lipid-raft fractions.

Acknowledgements

We thank Professor Simon Croft (London School of Hygiene and Tropical Medicine) for providing us with the WT and HePC-R lines of L. donovani and A. Bochot for supplying MCD. We are grateful to Françoise Huteau for her technical assistance, Jean-Christophe Jullian for his advice regarding NMR experiments and G. Barrant for critical reading of this manuscript prior to submission.

Funding

This work was supported by a grant from Institut Fédératif de Recherche (IFR 141) ‘Lipidomics in Leishmania’.
Sterol depletion impairs antileishmanial action of miltefosine

Transparency declarations

None to declare.

References