Interaction between Miltefosine and Amphotericin B: Consequences for Their Activities towards Intestinal Epithelial Cells and *Leishmania donovani* Promastigotes In Vitro[▽]

Cécile Ménez,¹ Marion Buyse,² Madeleine Besnard,¹ Robert Farinotti,²
Philippe M. Loiseau,³ and Gillian Barratt^{1*}

Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Univ. Paris-Sud 11, Faculté de Pharmacie, IFR 141, Châtenay-Malabry F-92296, France¹; Laboratoire de Pharmacie Clinique, UPRES 2706, Univ. Paris-Sud 11, Faculté de Pharmacie, IFR 141, Châtenay-Malabry F-92296, France²; and Chimiothérapie antiparasitaire, UMR CNRS 8076, Univ. Paris-Sud 11, Faculté de Pharmacie, IFR 141, Châtenay-Malabry F-92296, France³

Received 10 July 2006/Returned for modification 20 August 2006/Accepted 1 September 2006

The aim of this study was to evaluate the potential of a combination of two antileishmanial drugs, miltefosine (HePC) and amphotericin B (AMB), when administered by the oral route. Caco-2 cell monolayers were used as a validated in vitro model of the intestinal barrier and *Leishmania donovani* promastigotes as a model for evaluating the effect of the drug combination. Spectroscopic measurements demonstrated that HePC and AMB associate, leading to the formation of mixed aggregates in which AMB is solubilized as monomers. The incubation of the association of HePC and AMB with Caco-2 cell monolayers, at a concentration higher than 5 μ M, led to (i) a reduction of the HePC-induced paracellular permeability enhancement in Caco-2 cell monolayers, (ii) an inhibition of the uptake of both drugs, and (iii) a decrease in the transepithelial transport of both drugs, suggesting that a pharmacokinetic antagonism between HePC and AMB could occur after their oral administration. However, the combination did not exhibit any antagonism or synergy in its antileishmanial activity. These results demonstrated a strong physicochemical interaction between HePC and AMB, depending on the concentration of each, which could have important consequences for their biological activities, if they are administered together.

Leishmaniasis, tropical parasitic diseases caused by the protozoan parasite *Leishmania*, are one of the six major diseases in developing countries, according to the World Health Organization (WHO). Visceral leishmaniasis (VL), caused by *Leishmania donovani*, remains fatal if untreated. Traditionally, VL is treated with 4 weeks of injections of sodium stibogluconate, a pentavalent antimonial. However, resistance to this treatment has been observed in 37 to 64% patients in the current Indian epidemic zone (27). Parenteral amphotericin B (AMB) is used as a secondary agent and shows 95% effectiveness; however, its toxicity and the high cost of the well-tolerated liposomal complex, preclude its widespread use in developing countries. Recently, miltefosine (hexadecylphosphocholine [HePC]), an alkylphosphocholine, was licensed for use in the treatment of VL (Impavido), with the major advantage of being active orally. HePC has shown a 98% cure rate for VL patients with a dose of 2.5 mg/kg daily for 28 days (38).

One of the major barriers to successful treatment of these infections is the development of drug resistance. The widespread use of HePC as a single agent in India and its long clinical half-life of approximately 7 days might lead to rapid

emergence of resistance. Since HePC has only recently been introduced, clinical resistance has not yet been reported in the field but the ease with which resistant mutant parasites can be generated in the laboratory is a cause for concern (36). To slow down the potential emergence of resistant parasites and to prevent this novel oral agent from becoming obsolete quickly, it may be appropriate to use HePC in combination with other antileishmanial drugs. The development of a new orally effective bitherapy could open new perspectives for the treatment of VL, preventing and treating leishmaniasis without inconveniencing the patient, thus improving compliance and facilitating its use in developing countries.

An HePC-AMB bitherapy would be interesting for three reasons. (i) A potentiation of HePC activity in vivo was recently demonstrated when the drug was used in combination with AMB (37). (ii) We have recently demonstrated that HePC enhances the paracellular permeability of Caco-2 cell monolayers (25). This could allow small molecules to cross the intestinal barrier via the paracellular space and could therefore be exploited to improve the transport and the oral availability of AMB, a compound which has hitherto required intravenous administration, when the drug combination is used. (iii) HePC, an amphiphilic molecule composed of a zwitterionic head group and a C₁₆ hydrocarbon chain, is extremely water soluble and forms micelles with a critical micellar concentration (CMC) of 8 to 12 μ M (10, 22). The hydrophobic nature of AMB molecules would allow them to be solubilized in mixed micelles with this surfactant, as already observed with deoxycholate (Fungizone), lysophosphatidylcholine (21), lauryl su-

* Corresponding author. Mailing address: Faculty of Pharmacy, University of Paris-Sud, Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Tour D5, 2ème étage, 5 rue J.B. Clément, 92296 Châtenay-Malabry Cedex, France. Phone: 33 (0)1 46 83 56 27. Fax: 33 (0)1 46 61 93 34. E-mail: gillian.barratt@cep.u-psud.fr.

[▽] Published ahead of print on 11 September 2006.

crose, and palmitoyl mannose (18), thus providing a potential oral delivery system with rapid-self-emulsifying properties.

In this context, we have investigated the behavior of combinations of HePC and AMB in two in vitro models. The human-derived colon carcinoma epithelial cell line Caco-2 was grown on Transwell-clear polyester membranes and used as a validated intestinal transport model system (20), while *L. donovani* promastigotes were used to assess antiparasitic activity. The objectives of this study were (i) to investigate the physicochemical interactions between HePC and AMB through the spectroscopic properties of AMB, (ii) to evaluate the effects of HePC and AMB on each other's transport across the intestinal barrier, and (iii) to study the nature of the drug interaction towards *L. donovani* promastigotes.

MATERIALS AND METHODS

Materials. Unlabeled miltefosine (HePC) was from Cayman Chemical Company (Ann Arbor, Michigan). Solutions of HePC (10 mM) were freshly made in water or Krebs modified buffer immediately before each experiment. $[9,10\text{-}^3\text{H}]\text{HePC}$ was synthesized as described previously (12, 17) and stored in absolute ethanol at -20°C . AMB was from Bristol-Myers Squibb (Princeton, NJ). A stock solution of AMB at 10 mM was prepared in dimethyl sulfoxide (DMSO). $\text{D}\text{-}[^{14}\text{C}]\text{mannitol}$ (specific activity, 58 mCi/mmol) was purchased from Amersham Life Science (Buckinghamshire, United Kingdom). Ultima Gold liquid scintillation fluid was from Perkin Elmer Life Science Products (Boston, MA). Dulbecco's modified Eagle's medium, phosphate-buffered saline, fetal bovine serum, nonessential amino acid solution (10 mM, 100 $\times$), penicillin-streptomycin solution (10,000 units/ml penicillin and 10 mg/ml streptomycin), and trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA) were obtained from Invitrogen-Life Technologies. Transwell-clear polyester membranes (1-cm² surface area, 0.4- μm pore size) in 12-well plates and standard 12-well plates were purchased from the Costar Corning Corporation (NY). All other chemical products were from Sigma-Aldrich (St. Louis, MO). High-performance-liquid-chromatography (HPLC)-grade acetonitrile, tetrahydrofuran, triethylamine, and methanol (MeOH) used during the HPLC analysis were from Carlo Erba (Val de Reuil, France). Analytical-grade (USP-grade) DMSO was from Gaylord Chemical Corporation (Bogalusa, Louisiana). Triethylamine was from VWR International (Fontenay/Bois, France). Deionized water was obtained from a Millipore Milli-Q purification system (Millipore, Milford, MA).

Preparation of HePC-AMB mixtures. Micellar systems composed of AMB molecules incorporated into HePC micelles were prepared by dissolving AMB in a solution of HePC. AMB at 20 mM in DMSO was diluted in MeOH to 1 mM and added to the HePC solution in Krebs modified buffer under agitation. The mixtures were stirred thoroughly for 10 min before being added to the cells or parasites. The medium used for control incubations received the same amount of MeOH and DMSO, at a concentration not exceeding 0.5% of MeOH and 0.025% DMSO in the final medium.

Absorption and CD measurements. Studies of the spectral properties of AMB are able to give much useful information about the aggregation state of drug molecules in water (3, 5, 23) and their interactions with other compounds, such as lipids (24). They were therefore used to monitor the influence of HePC on the aggregation state of AMB and physicochemical interactions between the two. The absorption spectra of AMB were recorded using a Perkin-Elmer Lambda 11 UV-visible spectrophotometer, and circular-dichroism (CD) spectra of AMB were recorded with a Jasco J-810 dichrograph. $\Delta\epsilon$ is the differential molar dichroic absorption coefficient ($\text{M}^{-1}\text{cm}^{-1}$). Spectra were recorded within 30 to 45 min after preparation of the samples.

Caco-2 cell culture. Caco-2 cells (passages 45 to 65) were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 1% non-essential amino acid solution, 1% L-glutamine, and 1% penicillin-streptomycin mixture. Cells were kept at 37°C in 5% CO_2 and 95% humidity. Every week, cells were trypsinized and seeded at 5.10^4 cells per insert onto 12-well Transwell plates for transport studies and at 5.10^4 cells per well in standard 12-well plates for uptake studies. Cells were then grown in the plates for a minimum of 14 days and used for experimentation between days 14 and 21. The medium was changed daily.

The quality of the monolayers grown on the polyester membrane was assessed by measuring the paracellular transport of $[^{14}\text{C}]\text{mannitol}$ and the transepithelial electrical resistance using an EVOM epithelial tissue voltammeter (World

Precision Instrument, Sarasota, FL). For all the experimental conditions used, cell viability was checked using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay according to the procedure previously described (26). The experiment was accepted when the cell viability was more than 90%.

Transepithelial transport studies with Caco-2 cell monolayers. Transepithelial transport was studied with cells grown on permeable supports (Transwell) for 18 days. The transport medium consisted of Krebs modified buffer (5.4 mM KCl, 2.8 mM CaCl_2 , 1 mM MgSO_4 , 0.3 mM NaH_2PO_4 , 137 mM NaCl, 0.3 mM KH_2PO_4 , 10 mM glucose, and 10 mM HEPES-Tris [pH 7.4] or 10 mM MES [morpholineethanesulfonic acid]-Tris [pH 6.0]). The pH was adjusted to 6.0 in the apical compartment and to 7.4 in the basolateral compartment to ensure a proton gradient. The culture medium for the apical and basolateral compartments was removed by aspiration, and monolayers were washed three times with substrate-free transport medium at 37°C , 30 min before the beginning of the experiment. At time zero, buffer containing various compounds ($[^3\text{H}]\text{HePC}$ [0.5 μCi per well], $[^{14}\text{C}]\text{mannitol}$ [0.5 μCi per well], or AMB) was added to the apical compartment of the insert and the flux across Caco-2 cell monolayers was determined in the absence or presence of other drugs. The monolayers were kept at 37°C and 5% CO_2 and were continuously agitated on a shaker during the transport experiments. For HePC and mannitol transport, the amounts of radiolabeled compound transported across Caco-2 cell monolayers were determined by counting the samples in a Beckman LS 6000TA liquid scintillation counter. For studies of the transepithelial transport of AMB, the amounts of compound transported across Caco-2 monolayers were determined by HPLC. The apparent permeability coefficient P_{app} (cm/s) for the transported compounds was determined by the following equation: $P_{\text{app}} = (1/AC_0)(dQ/dt)$, where dQ/dt is the flux across the monolayer, A is the surface area of the Transwell membrane, and C_0 is the initial concentration of the compound in the donor compartment. Unless otherwise indicated, the P_{app} was determined in the apical-to-basolateral direction after a 3-h incubation.

Apical uptake of AMB and HePC by Caco-2 cells. Cellular uptake studies were performed with Caco-2 cell monolayers grown on 12-well dishes for 14 days. Before experiments, the culture medium was removed and cells were washed three times with substrate-free incubation medium at 37°C consisting of Krebs modified buffer (pH 6.0), as described previously. Apical uptake was started by the addition of $[^3\text{H}]\text{HePC}$ (0.1 μCi per well) and/or AMB in the presence or absence of other compounds dissolved in Krebs modified buffer (pH 6.0). At the end of the 1-h incubation period, the medium was immediately collected and cells were washed three times with ice-cold Krebs modified buffer at pH 6.0.

For HePC uptake measurement, cells were lysed in 1% Triton X-100 and processed for liquid scintillation counting. To determine AMB uptake, 0.75 ml of cold MeOH was added to the monolayer. After a 2-h incubation at 4°C , the cell extract was collected, vortexed vigorously, and centrifuged at $16,000 \times g$ for 20 min at 4°C . The clear supernatant was collected and processed for HPLC analysis.

Parasite strains and culture. Promastigote forms of a wild-type *L. donovani* (MHOM/ET/67/HU3/L82) clone were kindly provided by S. L. Croft (Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom) and were grown in M-199 medium supplemented with 40 mM HEPES, 100 μM adenosine, 0.5 mg/liter hemin, 10% heat-inactivated fetal bovine serum, and 50 $\mu\text{g}/\text{ml}$ gentamicin at 26°C in a dark environment. All experiments were performed with parasite cultures in the logarithmic phase of growth.

Assessment of drug interactions and isobologram construction. In order to determine the nature as well as the intensity of the drug interaction in its effect on *L. donovani* promastigote growth, a modified fixed-ratio isobologram method (14) using the MTT assay (26) was employed. Briefly, 50% inhibitory concentrations (IC_{50}) were determined on promastigotes after a 72-h exposure, as previously described (29). Fixed-ratio solutions at ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of HePC and AMB were prepared and then serially diluted seven times in twofold dilutions. The highest concentrations used were 40 μM for HePC and 0.2 μM for AMB. The fractional inhibitory concentrations (FICs) were calculated as described previously (4) and are defined as $\text{FIC} = (\text{IC}_{50})_{\text{XY}}/(\text{IC}_{50})_{\text{X}}$, where $(\text{IC}_{50})_{\text{X}}$ is the IC_{50} value for drug X acting alone, and $(\text{IC}_{50})_{\text{XY}}$ is the IC_{50} for the same drug in the presence of a suboptimal concentration of drug Y. FICs and sum FICs (ΣFICs [$\text{FIC}_{\text{HePC}} + \text{FIC}_{\text{AMB}}$]) were calculated for all fixed-ratio solutions. FICs were used to construct classical isobolograms, as described previously (19), and mean ΣFICs were used to define the nature of the interaction. Synergy was defined as an FIC of ≤ 0.5 ; indifference was defined as an FIC of > 0.5 but ≤ 4 , whereas antagonism was defined as an FIC of > 4 .

Quantitative analysis of AMB by HPLC. AMB concentrations in the samples were measured by a sensitive and specific HPLC assay with UV absorbance

FIG. 1. Influence of different HePC concentrations on the UV-visible absorption (A) and circular dichroism (B) spectra of AMB. All spectra are shown at a constant concentration of AMB (10 μ M) in Krebs modified buffer. (a) AMB alone in incubation medium. HePC/AMB mixtures were studied at HePC/AMB molar ratio of 5 (b), 20 (c), and 50 (d). The spectra are representatives of three repeated experiments. Inset, representative spectrum for the association of AMB at 5 μ M and HePC at 20 μ M, the concentrations used in biological experiments. $\Delta\epsilon$, differential molar dichroic absorption coefficient.

detection at 409 nm, corresponding to the maximum absorbance for AMB. A Breeze HPLC system (Waters) consisting of a binary HPLC 1525 model pump, a UV-visible spectrophotometric detector (Dual λ Absorbance Detector 2487), and an integrator was used at an ambient temperature and a mobile-phase flow rate of 1.0 ml/min. The chromatographic separation was performed on a Hypersil Shandon C₁₈ column (150 mm $\times$ 4.6 mm; inside diameter, 5 μ m), using acetonitrile-tetrahydrofuran-triethylamine 0.1% in water at pH 5.2 as the mobile phase. A sample volume of 100 μ l was injected. A standard solution of AMB was prepared in DMSO and then diluted to a concentration of 200 μ M in MeOH. The working-standard solutions for constructing calibration curves and for assay validation were obtained by dilution of the stock solution in DMSO-MeOH with MeOH or Krebs buffer. Under these conditions, the retention time for AMB was 6 min. Validation of the assay showed that the calibration curve was linear ($R^2 = 0.997$) over the concentration range of 10 to 20,000 nM of AMB, with detection and quantitation limits of 5 and 10 nM of AMB, respectively. The HPLC assay was found to be specific for AMB without interference from the components of the transport buffer or HePC.

Statistics. All experiments were conducted at least in triplicate, and results are expressed as means $\pm$ standard deviations (SDs). Statistical analysis was performed using one-way analysis of variance with a Mann-Whitney posttest for double comparisons or a Student's *t* test (GraphPad Instat, San Diego, CA). *P* values of <0.05 were accepted as statistically significant.

RESULTS

Spectroscopic measurements of HePC-AMB interactions.

UV-visible and CD spectroscopy techniques were particularly appropriate for characterizing our systems since they have been shown to be very sensitive to the aggregation state of AMB as well as to the interaction of the drug with its molecular environment (1, 6). Figure 1 presents the UV-visible and CD spectra for AMB at a constant concentration of 10 μ M (i.e., above its CMC of about 10^{-7} M) alone in solution or in mixtures with HePC at various concentrations (HePC/AMB molar ratios of 5, 20, or 50). It should be noted that the concentration of HePC in these mixtures was also above its CMC, which has been reported to be around 10 μ M (10). The UV-visible absorption spectrum of AMB alone in water was comparable to that already observed in the literature, showing the presence of both aggregated and monomeric forms of AMB. In particular, it showed an absorption band at about 409 nm, characteristic of AMB in monomeric form, and an absorption band around 328 nm, characteristic of AMB in aggregated

form (2). The addition of increasing concentrations of HePC to the medium led to changes in the AMB absorption spectrum as shown in Fig. 1A: (i) there was a shift of the peak from 409 to 414 nm, demonstrating the interaction of AMB with HePC, and (ii) at higher HePC/AMB ratios, there was a decrease of the intensity of the band around 330 nm and a concomitant increase of the intensity of the band at 414 nm, demonstrating a reduction of the aggregated form and an increase in the monomeric form of AMB. This phenomenon could be attributed to the formation of a complex between AMB and HePC.

The CD spectrum of AMB alone in the medium exhibited a strong dichroic doublet centered around 340 nm (Fig. 1B), characteristic of self-associated AMB (6). Figure 1B shows that the addition of HePC also led to modifications in the CD spectra of AMB: (i) the intense dichroic doublet was shifted from 340 to 334 nm, and (ii) at higher HePC/AMB molar ratios, the dichroic doublet of AMB decreased, almost disappearing for an HePC/AMB molar ratio of 50. It is well established that a weak CD intensity for all wavelengths is characteristic of monomeric AMB (7). The CD results were therefore in agreement with those of UV-visible measurements: HePC induced a reorganization of the AMB aggregates and their disaggregation at higher concentrations.

It has to be noted that these changes in the spectroscopic properties of AMB did not occur when HePC was added below its critical micellar concentration, at 4 and 8 μ M (data not shown), strongly arguing for a micellar solubilization of the AMB aggregates by HePC micelles. Furthermore, the concentrations used for spectroscopic measurements were relatively high, due to the limited sensitivity of the instruments. In subsequent studies using the Caco-2 cell model, the concentrations used were, unless otherwise indicated, 20 μ M for HePC and 5 μ M for AMB. The spectrum for the association of these concentrations is presented in the inset of Fig. 1A. It was similar to the spectrum obtained with a 5:1 ratio of HePC to AMB at 10 μ M AMB, showing that, above the CMC of HePC, the interaction depended on the ratio rather than the concentration.

FIG. 2. Effect of AMB concentration (1, 5, 50, and 100 μ M) on HePC-induced changes in the permeability of mannitol as a marker of paracellular transport across Caco-2 cell monolayers. Results shown are the ratios of P_{app} calculated from HePC-treated cells to P_{app} calculated from untreated cells (control). All measurements are expressed as means $\pm$ SDs ($n = 9$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control values.

Effects of AMB on HePC-induced paracellular permeability enhancement. We have previously demonstrated that HePC treatment increases the paracellular permeability of Caco-2 cell monolayers (25). Since the aim of the study was to investigate the possibility of coadministering HePC and AMB orally, we investigated the influence of various concentrations of AMB on this HePC-induced tight-junction opening (Fig. 2). Mannitol was used as a marker for paracellular permeability. As expected, after apical treatment of Caco-2 monolayers with 20 μ M HePC for 3 h, the P_{app} of mannitol was significantly increased approximately eightfold (from $0.89 \times 10^{-6} \pm 0.17 \times 10^{-6}$ to $7.47 \times 10^{-6} \pm 1.69 \times 10^{-6}$ cm/s), confirming the opening of the tight junctions. A control with AMB treatment alone showed that AMB did not enhance the permeability of mannitol with the time and doses used in the study (1 to 100 μ M) (data not shown). When HePC was coincubated with AMB at a low concentration (1 or 5 μ M), the mannitol P_{app} was also significantly increased compared with the control value, indicating that the HePC treatment still induced an opening of the tight junctions. However, when HePC was coincubated with a higher dose of AMB (50 or 100 μ M), the mannitol P_{app} was not significantly different from the control value, suggesting that AMB inhibited the HePC-induced paracellular permeability enhancement.

FIG. 3. Effect of coincubation of HePC and AMB on their transepithelial transport (A and B) and on their apical uptake by Caco-2 cell monolayers (C and D). Apical-to-basolateral transport of [3 H]HePC (20 μ M) (A) or AMB (5 μ M) (B) was measured in the absence (control) and in the presence of various concentrations of AMB or HePC, respectively. (C) The effect of coincubation of AMB with HePC was investigated by adding AMB (5, 50, or 100 μ M) to [3 H]HePC (20 μ M) during the HePC uptake experiment. (D) The effect of coincubation of HePC with AMB was investigated by adding HePC (5, 50, or 100 μ M) to AMB (5 μ M) during the AMB uptake experiment. Results are the means $\pm$ SDs for three determinations with three different monolayers ($n = 9$). For uptake determination, results are calculated as percentages of the control values (single agent). *, $P < 0.05$; **, $P < 0.01$ versus control values.

FIG. 4. Effect of preincubation of HePC on AMB transport across Caco-2 cell monolayers. Cells were preincubated for 2 h with HePC (5, 50, or 100 μ M) in the apical compartment or in Krebs modified buffer (pH 6.0) as a control, and the P_{app} values for AMB (5 μ M) were calculated after a 2-h incubation. All measurements are expressed as means $\pm$ SDs for three determinations with three different monolayers ($n = 9$). **, $P < 0.01$; ***, $P < 0.001$ versus control values.

Coadministration of HePC and AMB as a complex. (i) Transepithelial transport of HePC and AMB in Caco-2 cell monolayers. Transport of HePC and AMB across Caco-2 cell monolayers was investigated for the drugs added alone and in various combinations (Fig. 3A and B). The P_{app} values for HePC and AMB alone in media were found to be $2.83 \times 10^{-6} \pm 0.34 \times 10^{-6}$ cm/s (Fig. 3A) and $1.69 \times 10^{-10} \pm 0.36 \times 10^{-10}$ cm/s (Fig. 3B), respectively.

In the case of coinubation of HePC and AMB, a significant decrease in the transport of both molecules across Caco-2 cell monolayers was observed, down to $1.47 \times 10^{-6} \pm 0.11 \times 10^{-6}$ cm/s for HePC in the presence of 100 μ M AMB and $0.83 \times 10^{-10} \pm 0.19 \times 10^{-10}$ cm/s for AMB in the presence of 100 μ M HePC. Interestingly, instead of increasing the transport of AMB through an opening of the tight junctions by HePC, the coadministration of HePC and AMB seemed to inhibit the transport of both drugs across the Caco-2 cell monolayer. This suggests a strong interaction between the two drugs and a "pharmacokinetic antagonism."

(ii) Uptakes of HePC and AMB by Caco-2 cells. To explain the reductions in HePC and AMB transport that occurred when the drugs were administered together, the uptakes of drugs from the apical sides of Caco-2 cells were investigated. The results are presented in Fig. 3C and D. When HePC was coinubated with AMB at a concentration of 50 or 100 μ M, its cellular uptake from the apical sides of Caco-2 cell monolayers was significantly reduced (Fig. 3C). Similarly, in the case of coinubation of AMB with increasing concentrations of HePC, the apical uptake of AMB was markedly reduced regardless of the HePC concentration (Fig. 3D). A control using testosterone, a lipophilic marker, showed that HePC or AMB had no effect on the cellular uptake of this marker, further suggesting that the interactions observed between HePC and AMB on their apical uptakes may be specific (data not shown).

Sequential administration of HePC and AMB. (i) Transepithelial transport of HePC and AMB in Caco-2 cell monolayers. Since we demonstrated that coadministration of HePC and AMB did not enhance the transepithelial transport of AMB in Caco-2 cells, we investigated the effect of the preexposure of Caco-2 monolayers to HePC on the transport of AMB across the intestinal barrier. Indeed, we have previously demonstrated that there was a latent period before the maximal effect of HePC on the opening of the Caco-2 tight-junction complexes was manifested (25).

Figure 4 shows that a sequential application, with a 2-h preexposure to HePC concentrations higher than 5 μ M, led to a significant enhancement of AMB transport across the intestinal barrier in a concentration-dependent manner (from $1.43 \times 10^{-6} \pm 0.18 \times 10^{-6}$ to $3.00 \times 10^{-10} \pm 0.30 \times 10^{-10}$ cm/s when Caco-2 monolayers were exposed to 100 μ M HePC). This result demonstrated that, when the HePC molecule which opens the tight junctions was removed from the extracellular medium before adding AMB, the paracellular transport of the latter was enhanced. This observation confirmed the hypothesis of the physicochemical interaction between the two drugs when they were coadministered. It should be noted that a preexposure to AMB did not affect HePC transport across Caco-2 monolayers (data not shown).

(ii) Uptake of HePC and AMB by Caco-2 cells. The influence of the sequential administration of the two drugs on their apical uptake by Caco-2 cells was also investigated. To preload the cells with drugs, they were preincubated with HePC or AMB at 100 μ M for 2 h, before measuring the uptake of the partner drug. The results in Table 1 show that after a preexposure with 100 μ M of HePC for 2 h, AMB uptake was not modified, whereas the preincubation with 100 μ M AMB led to a significant reduction of HePC uptake by around 20%. This result demonstrated that the order of the sequential administration was important. Apparently, the interaction of AMB with cell membrane was not modified by HePC preincubation, suggesting that HePC, when present in the cell membrane, did not interfere with AMB association. On the other hand, the presence of AMB-sterol complexes in the Caco-2 cell membrane seemed to partially inhibit the insertion of HePC into the membrane.

In vitro drug interactions against *L. donovani* promastigotes. In vitro interactions were assessed using a modified fixed-ratio isobologram method. The IC_{50} (concentrations provoking 50% inhibition of growth of promastigotes) against *L. donovani* obtained in this study for HePC and AMB were $7.2 \pm 0.9 \mu$ M

TABLE 1. Effect of preexposure to either HePC or AMB on the apical uptake of the other drug by Caco-2 cell monolayers

Drug	Condition	Uptake ^a (% of control value)
AMB	Control	100 $\pm$ 7.6
	Preexposure with HePC (100 μ M)	109.8 $\pm$ 5.9
HePC	Control	100 $\pm$ 2.6
	Preexposure with AMB (100 μ M)	81.1 $\pm$ 8.5 ^b

^a Cellular accumulations are expressed as means $\pm$ SDs for three determinations with three different monolayers ($n = 9$) and calculated as percentages of control values (for the drug in question alone).

^b $P < 0.05$ versus control value.

FIG. 5. A representative isobologram of in vitro interactions between HePC and AMB against *Leishmania donovani* promastigotes. The FICs, calculated as described in Materials and Methods, are plotted. The bold line corresponds to the predicted positions of the experimental points for a simple additive effect.

and 71.6 ± 6.3 nM, respectively, and were comparable to those recently reported in the literature (8, 13). A representative isobologram for the HePC-AMB combination is shown in Fig. 5. The combination of HePC with AMB displayed convex curves consistently at all dose levels, suggesting a gradual decrease of the antileishmanial activity of HePC with increasing doses of AMB (4). However, the interaction of HePC with AMB was found to be indifferent, with mean Σ FICs of 1.429 ± 0.19 . A similar result was obtained previously (37), in a study in which AMB was administered as the commercial formulation Fungizone (mixed micelles with deoxycholate).

DISCUSSION

The aim of this study was to evaluate the possibility of developing a combination of two antileishmanial drugs, HePC and AMB, for oral administration. This sort of formulation could be useful for preventing the emergence of drug resistance, increasing efficacy, or shortening the course of treatment of visceral leishmaniasis infection. However, the development of an oral AMB formulation remains a challenge. A number of studies have reported that AMB is poorly absorbed from the gastrointestinal tract (33) and is therefore routinely administered intravenously. The low P_{app} values for AMB across Caco-2 cell monolayers found in our study are in accordance with these observations. The poor oral bioavailability of AMB is due to low gastrointestinal permeability and poor solubility leading to slow dissolution from precipitated AMB in the lumen (33). Improved gastrointestinal absorption has therefore been achieved by increasing the dissolution rate of AMB by incorporation into micellar or mixed micellar systems containing bile acids and phospholipids (9) or association with a lipid-based delivery vehicle such as Peceol (32) or cochleates (35), but none of these formulations are licensed for systemic antifungal or antiparasitic treatment.

In this study, the modifications in the spectral properties of AMB observed in the presence of HePC above its CMC indicate the formation of complexes, as already noted for other

AMB-lipid interactions (15). This results in a significant decrease in the degree of aggregation of AMB and an increase in the proportion of monomeric AMB with increasing HePC concentration, suggesting that this AMB monomer is associated with HePC micelles. Micellar solubilization of AMB has already been observed with deoxycholate (Fungizone), lysophosphatidylcholine (21), lauryl sucrose, and palmitoyl mannose (18). HePC-AMB association within a micellar system could provide a potential oral delivery system with rapid-self-emulsifying properties. We therefore investigated the behavior of HePC and AMB alone and in association in the in vitro intestinal model of Caco-2 cell monolayers.

We demonstrated in this study that the association of HePC and AMB in the incubation medium, at an AMB concentration higher than $5 \mu\text{M}$, modified the effects of HePC, including a reduction in its ability to enhance paracellular permeability as previously described (25). Furthermore, the presentation of HePC and AMB at the same time was found to reduce the uptake of both at the apical sides of Caco-2 cell monolayers and to decrease the transepithelial transport of both, suggesting that a pharmacokinetic antagonism between HePC and AMB could occur after their oral administration.

Sequential administration of the two agents led to different results: pretreatment of the monolayers with HePC led to a dose-dependent increase in AMB permeability, which could be attributed to the ability of the ether lipid to open epithelial tight junctions (25), and did not affect the uptake of AMB into cells. Therefore, it would appear that the antagonism between HePC and AMB observed on simultaneous administration is due to the formation of HePC-AMB mixed micellar complexes, which prevents their entry into the plasma membrane and reduces their uptake and transepithelial transport. We therefore hypothesize that free HePC and AMB molecules rather than the ether lipid-AMB complex can penetrate into the plasma membrane.

Some interaction between the two drugs may also occur at the level of the intestinal cell membrane, because HePC uptake was also reduced by preincubation with AMB. Both of these drugs are known to interact with the plasma membrane of mammalian cells: AMB forms transmembrane pores (34), whereas HePC is accumulated in the bilayer (39). In particular, they may compete for binding to membrane sterols. It is well known that the target of AMB is membrane sterols, leading to the formation of an AMB-sterol complex followed by transmembrane channels (16). More recently, a strong interaction between HePC and membrane sterols has been demonstrated (30). Formation of a stable HePC-cholesterol complex in which two HePC molecules and one molecule of cholesterol are strongly bound together has been identified (31). Furthermore, a correlation between the membrane cholesterol content and the incorporation of HePC as well as the regulation of its biological activities has been suggested (11), and more recently, the incorporation of HePC preferentially into cholesterol-rich microdomains called "rafts" (40) has been described.

In our study, the effect of the drug combination against *L. donovani* promastigotes in vitro was found to be indifferent. A similar result was described in a recent study (37); however, the experiments presented were carried out using the commercial form of AMB, as mixed micelles with deoxycholate, which could have affected its interactions with HePC, so it was nec-

essary to repeat the experiment using pure AMB. This lack of interaction and the fact that AMB exhibited the same activity on *L. donovani* wild-type and HePC-resistant clones where the HePC transporter is not functional suggest that AMB did not interfere with the *L. donovani* HePC transporter which has been recently identified (28). However, a pharmacological interaction between HePC and AMB in vivo has been shown by Seifert and Croft (37). In this case, AMB (amphotericin B deoxycholate; Fungizone) was given by the intravenous route while HePC was administered orally, thus avoiding any interactions at the level of the intestinal lumen or the enterocytes. Under these conditions, potentiation of HePC activity by AMB was observed.

We have demonstrated a twofold enhancement of the gastrointestinal membrane permeability of AMB after preincubation with HePC. This result suggests that AMB could cross the intestinal barrier via the paracellular route, since we have demonstrated previously that HePC opens Caco-2 tight junctions (25). However, the enhancement of AMB absorption should be investigated in vivo with measurement of the AMB concentrations in plasma after sequential administration to ensure that an effective concentration of the drug can be achieved, followed by studies of its efficacy in the *L. donovani*/BALB/c mouse model.

In conclusion, this study demonstrates strong physicochemical and biological interactions between two antileishmanial agents, HePC and AMB, depending on the concentrations of each. These findings have important biological consequences, considering that these in vitro data suggest pharmacokinetic antagonism. Further investigation is required to clarify the actual mechanism underlying the interaction between the two drugs and to investigate the correlation between the physicochemical interactions and the biological effects of the HePC-AMB complex.

ACKNOWLEDGMENTS

We are grateful to Laurent Massias for providing the HPLC assay protocol. We also thank Hélène Chacun for valuable help in radioactivity studies. We gratefully acknowledge Françoise Huteau for help with parasite culture and isobologram experiments and Christophe Dugave for the [³H]HePC synthesis.

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