available at www.sciencedirect.com

SCIENCE DIRECT®

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

Modulation of intestinal barrier properties by miltefosine

Cécile Menez [a,*], Marion Buyse [b], Hélène Chacun [a], Robert Farinotti [b], Gillian Barratt [a]

[a] Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France

[b] Laboratoire de Pharmacie Clinique, UPRES 2706, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France

ARTICLE INFO

Article history:
Received 28 September 2005
Accepted 4 November 2005

Keywords:
Miltefosine
Hexadecylphosphocholine
Caco-2
Tight junctions
Paracellular permeability
P-glycoprotein

ABSTRACT

Miltefosine (hexadecylphosphocholine, HePC) is the first effective oral agent for the treatment of visceral leishmaniasis. This study aimed to determine whether this oral administration alters the integrity and transport capacities of the intestinal barrier. The objectives of this study were: (i) to evaluate the cytotoxicity of HePC, (ii) to investigate the effects of HePC on paracellular and transcellular transport and (iii) to investigate the influence of HePC on three major transporters of the intestinal barrier, namely, P-glycoprotein, the human intestinal peptide transporter (PepT-1) and the monocarboxylic acid transporter (MCT-1) in Caco-2 cell monolayers, used as an in vitro model of the human intestinal barrier. We show that HePC reduced the transepithelial electrical resistance and increased D-[^{14}C]mannitol permeability in a dose-dependent manner but had no effect on [^{3}H]testosterone permeability, demonstrating that HePC treatment enhances paracellular permeability via an opening of the tight junction complex without affecting the transcellular route. Morphological studies using confocal fluorescence microscopy showed no perturbation of the normal distribution of ZO-1, occludin or E-cadherin but revealed a redistribution of the tight junction-associated protein claudin-1 and the perijunctional actin after incubation with HePC. Finally, HePC was found to inhibit the intestinal P-glycoprotein in the Caco-2 cell model after a single short exposure. These results suggest that HePC could modify the oral bioavailability of other therapeutic compounds absorbed via the paracellular route or which are substrates of the intestinal P-glycoprotein.

1. Introduction

Miltefosine (hexadecylphosphocholine, HePC), an alkyl-lysophospholipid (ALP), is the first orally effective drug against visceral leishmaniasis (VL) and has proved to be highly effective and less toxic than current therapies. It has shown a 98% cure rate of VL patients during a phase III clinical trial in India [1]. Following registration in India in 2002 for the treatment of VL, HePC is about to play an essential role in the control and treatment of this endemic disease [2]. Indeed, this oral administration is a major advantage over the currently recommended antileishmanial drugs that require parenteral administration. Although data on the remarkable activity after oral administration exist, little is known about the mechanisms of action of HePC and other ALPs. It has been reported that ALPs act primarily at the cell membranes [3] and alter their physicochemical properties [4]. Moreover, the effect of HePC on the intestinal barrier has not yet been fully investigated. This question is all the more relevant since it has been claimed that ALPs could enhance in vitro epithelial permeability of human colorectal cancer cell monolayers T84 [5] and Madin-Darby canine kidney (MDCK) cell monolayers [6]. Furthermore, previous studies performed with similar phospholipid-like compounds: dodecylphosphocholine (DPC)

* *Corresponding author*. Tel.: +33 1 46 83 56 27; fax: +33 1 46 61 93 34.
E-mail address: cecile.menez@cep.u-psud.fr (C. Menez).
0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.11.008

[7], 2-alkoxy-3-alkylamidopropylphosphocholines [8] or 3-alkylamido-2-alkoxypropylphosphocholines [9], have shown that they are able to enhance the transport of hydrophilic compounds across Caco-2 cell monolayers by modulating tight junctions. These authors claimed that several lysophosphatidylcholines could be used as enhancers of paracellular permeability. In fact, several phospholipids and phospholipid-like agents such as lysophosphatidylcholines or lysolecithin increase the paracellular permeability of hydrophilic compounds across various epithelia [10,11]. Even if the mechanism is still unclear, lipid surfactants can increase the permeability of epithelial barriers in a concentration-dependent manner [12] and it has been suggested that these phospholipid derivatives also increase the paracellular permeability of hydrophilic compounds by modulation of tight junctions [7,13,14].

Furthermore, it has also been reported that phospholipid-like agents such as non-ionic surfactants (i.e. Tween 80, Cremophor EL and glycerol esters) with membrane fluidity modulating activity were able to alter the activity of several specific transporters [15–17] and inhibit efflux systems in epithelial models.

Taken together, these results suggest that HePC could modify the transport properties of the intestinal epithelial barrier and thus influence – and perhaps improve – oral absorption of other therapeutics agents delivered via the oral route.

The aim of our present experiments was to investigate the action of HePC on the intestinal epithelium barrier. The human colon carcinoma cell line Caco-2 grown on Transwell®-clear polyester membranes was used as a validated intestinal transport model system [18,19]. The three objectives of this study were: (i) to determine HePC cytotoxicity towards Caco-2 monolayers, (ii) to evaluate the effects of HePC on paracellular and transcellular passive transport across Caco-2 cell monolayers and (iii) to establish its influence on three active membrane transporters, namely the P-glycoprotein (P-gp), the human intestinal peptide transporter (PepT-1) and the monocarboxylic acid transporter (MCT-1).

2. Materials and methods

2.1. Materials

D-[^{14}C]mannitol (specific activity 58 mCi/mmol) was purchased from Amersham Life Science (Buckinghamshire, UK), [^{3}H]testosterone (specific activity 78.5 Ci/mmol), [^{3}H]digoxin (specific activity 23.4 Ci/mmol) and Ultima Gold™ liquid scintillation were from Perkin Elmer Life Science Products (Boston, USA). N-[1-^{14}C]Butyric acid, sodium salt (specific activity 56 mCi/mmol) was from MP Biomedicals Inc. (CA, USA), [^{14}C]Gly-Sar (specific activity 49.94 mCi/mmol) was from New England Nuclear (Boston, MA, USA). HePC was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Solutions of HePC (10 mM) were freshly made in water or in Krebs modified buffer immediately before each experiment. Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), non-essential amino acids solution (NEAA 10 mM, 100×), penicillin–streptomycin solution (10,000 units/ml penicillin and 10,000 μg/ml of streptomycin), trypsin–EDTA solution (0.05% trypsin, 0.53 mM EDTA) were obtained from Invitrogen-Life Technologies. Transwell®-clear polyester membranes 12-well (1 cm^2 surface area, 0.4 μm pore size), Transwells® 24-well (6.5 mm diameter, 0.4 μm pore size), 6-, 12- and 96-well plates were purchased from the Costar Corning Corporation (NY, USA). Cytotoxicity Detection Kit (LDH) was from Roche Diagnostics (Meylan, France). Methylthiazoletetrazolium (MTT), sodium butyrate, phalloidin–tetramethylrhodamine (phalloidin TRITC labeled), glycyl-sarcosine (Gly-Sar) were from Sigma–Aldrich (St. Louis, MO, USA). Testosterone was from Fluka Chemika (Buchs, Switzerland). Antibodies directed against tight junction proteins, anti-mouse and anti-rabbit IgG antibodies (FITC-labeled) were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA).

2.2. Cell culture

Caco-2 cells (passages 45–65) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 1% non-essential amino acids, 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 5×10^3 cells per well onto 96-well cluster trays for cytotoxicity studies, at 5×10^4 cells per insert onto Transwells® 12-well for transport studies and at 2×10^4 cells per insert onto Transwells® 24-well for immunohistochemistry. 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.

2.3. Cytotoxicity assays

2.3.1. Cell viability: the MTT assay

The cell viability in presence of HePC was evaluated using the MTT colorimetric assay. Caco-2 cells seeded in 96-well culture plates were washed three times with PBS at 37 °C. Subsequently, increasing concentrations of HePC were added to the cells, PBS and DMSO were used as negative and positive controls, respectively. Caco-2 cells were further incubated at 37 °C for 2, 6 or 24 h. Thereafter, cell viability was determined by the MTT test according to the procedure described by Mosmann [20]. Briefly, 10 μl of MTT solution at 5 mg/ml in PBS were added to each well and plates were incubated at 37 °C for 4 h. Medium was removed and 100 μl of acid–isopropanol (0.04N HCl in isopropanol) and 20 μl of DMSO were added to each well and mixed thoroughly to completely dissolve the dark blue crystals. The optical density values were measured at 570 nm using a multiwell-scanning spectrophotometer.

2.3.2. Release of lactate dehydrogenase

The effect of HePC upon cell integrity was also determined by measurement of lactate dehydrogenase (LDH)-release as a marker of cell membrane damage. Cell monolayers grown on 96-well dishes were incubated with increasing concentrations of HePC. After 2, 6 and 24 h of exposure 100 μl samples were withdrawn and analyzed for LDH content. In control experiments, the monolayers were incubated with PBS or 1% Triton X-100. Afterwards, results were normalized to 0% and 100% LDH release, respectively.

2.4. Transport studies

Transepithelial transport was studied with cells grown on permeable supports (Transwell®) for 18 days. The transport medium consisting 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/Tris (pH 6.0). The pH was adjusted to 6.0 in the apical compartment and to 7.4 in the basolateral compartment. The culture medium of apical and basolateral compartment 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 0, buffer containing radiolabeled compound ([^{14}C]mannitol, [^{3}H]testosterone, [^{14}C]butyrate, [^{14}C]Gly-Sar or [^{3}H]digoxin) was added on the apical (0.5 ml) or basolateral (1.5 ml) compartment of the insert and the flux across Caco-2 cell monolayers was determined in the absence or presence of various concentrations of HePC. The monolayers were continuously agitated on a shaker during the transport experiments. The amount of radiolabeled compound transported across Caco-2 cell monolayers was determinated by counting the samples in a Beckman LS 6000TA liquid scintillation counter. At the end of the experiment, uptake of the radiolabeled compound into cell monolayer was determinated. For this, monolayers were washed three times with ice-cold buffer, solubilized with Triton X-100 at 1% and processed for scintillation counting.

The apparent permeability coefficient, P_{app} (cm/s) for the radiolabeled compounds were determined by the following equation: $P_{app} = (1/AC_0)(dQ/dt)$, 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 radiolabeled compound in the donor compartment.

2.5. Measurement of transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) is an index of confluence and integrity of monolayers [19]. The TEER value was measured at the beginning of transport experiments with an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, Sarasota, FL) to check the integrity of monolayers. Monolayers that displayed a TEER of 200–300 Ω cm^2 were used in the experiments. TEER measurements were also performed to evaluate the effect of HePC on tight junctions and on paracellular permeability. Thus, monolayers were treated on the apical side with various concentrations of HePC dissolved in Krebs modified buffer (pH 6). Measurements were performed at 37 °C and results were expressed as relative resistance (percentage of TEER in presence versus in absence of treatment).

2.6. Recovery experiments

In order to determine whether the effect of HePC on the intestinal barrier permeability was reversible or irreversible, cell monolayers were preincubated with HePC for 2 h, washed three times with Krebs buffer and then incubated with [^{14}C]mannitol (0.1 μCi per well) in fresh medium for 24 h. A control was performed with cells not treated with HePC. TEER was measured at the end of the experiment to check the integrity of the cell monolayer.

2.7. Immunohistochemistry

Caco-2 cell were grown on Transwell® for 18 days. Cell monolayers were incubated with HePC at 50 μM for 2 h. The medium was removed and the monolayers were washed three times with PBS at room temperature. The cells were fixed with 3% paraformaldehyde for 15 min, rinsed three times with PBS, and then permeabilized with 0.2% Triton X-100 for 10 min. The filter was cut out and after three washes, cells were blocked with PBS containing 1% BSA. After further rinsing, the cell monolayer was incubated with PBS containing 0.2% gelatin and 0.008% saponin. The cells were then incubated for 20 min with rhodamine–phalloidin diluted in PBS containing 1% BSA to reveal filamentous actin. The primary antibody (mouse monoclonal antibody against E-cadherin, ZO-1 and occludin, rabbit polyclonal antibody against claudin 1), diluted at 1:200 in PBS containing 0.2% gelatin, was applied to the cell monolayers and incubated for 1 h. After several washes with PBS/0.2% gelatin, cells were incubated for 45 min with the FITC-labeled goat anti-mouse or anti-rabbit IgG antibody, diluted at 1:200 in PBS/0.2% gelatin. After several rinses with PBS/0.2% gelatin, filters were mounted in glass slides and examined using a Zeiss confocal microscope.

2.8. Western blot analysis

For total protein extraction, Caco-2 cells pellets were homogenized at 4 °C in lysis buffer (Tris 10 mM, EDTA 5 mM, NaCl 126 mM, Triton 1% and SDS 0.1%) supplemented with 100 μmol/l aprotinin, leupeptin and pepstatin. The homogenates were centrifuged at 12,000 × g for 20 min at 4 °C and the supernatants collected for Western blot analysis. Proteins were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Briefly, 20 μg of proteins were solubilized in electrophoresis sample buffer containing β-mercaptoethanol, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gel (Bio-Rad) and transferred to nitrocellulose membranes. The blots were blocked 1 h in TBS buffer containing 0.1% Tween 20 (TTBS) and 10% non-fat dry milk. After washing with TTBS, the blots were incubated overnight at 4 °C with a 1:100 dilution of monoclonal antibody C219 (Dako, Glostrup, Denmark). After washing five times for 10 min in TTBS, they were further incubated 1 h at room temperature with an anti-mouse horseradish peroxidase-conjugated antibody diluted 1:10,000 (Amersham, UK). The membranes were washed four times for 10 min in TTBS and then revealed using a chemiluminescence system (ECL, Amersham Biosciences, UK). The intensity of the bands was quantified using Scion Image (NIH, Scion Corporation, Bethesda, USA).

2.9. Statistics

Results are expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using one-way analysis of

variance (ANOVA) with a Mann–Whitney post-test for double comparisons (GraphPad Instat, San Diego, CA). Statistical significance was accepted as $p < 0.05$.

3. Results

3.1. Effects of HePC on apical membrane integrity and cell viability

The aim of these studies was to determine subtoxic concentrations of HePC, which could be used in the subsequent studies on Caco-2 cell monolayers. The cytotoxicity of HePC towards Caco-2 cells was examined using the MTT assay and LDH release from Caco-2 cells. The dose-dependent viability of Caco-2 cells treated with HePC for 2, 6 and 24 h is presented in Fig. 1. These results showed that HePC had cytotoxic effects in a concentration and time-dependent manner. A 2-h treatment with HePC up to 200 μM induced no significant mitochondrial toxicity towards Caco-2 cells (more than 90% cell viability); however significant LDH release was observed. This result suggests that HePC could cause rapid membrane damage, without inducing a subsequent decrease in cell viability. A 6-h exposure to HePC led to a marked decrease in cell viability at concentrations above 50 μM and a 24-h exposure resulted in a more pronounced decrease in cell viability and a significant LDH leakage for all doses studied. The effective LD_{50} values (concentration that causes 50% lethality) at 2, 6 and 24 h were approximately 550, 140, and 75 μM, respectively. Subsequent studies were performed at HePC concentrations that gave more than 80% cell viability and moderate LDH release.

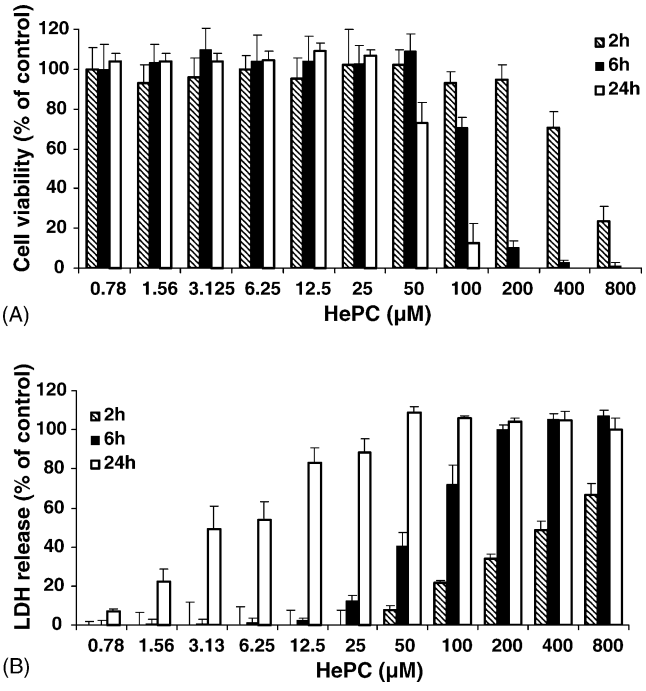

Fig. 1 – Cytotoxic effects of HePC on Caco-2 cell monolayers as a function of time and concentration. Cytotoxicity was measured by the MTT assay (A) and LDH release (B) following 2, 6 or 24 h incubation at 37 °C, 5% CO_2 and 95% relative humidity. For the MTT assay, PBS and DMSO were used as positive (100% cell viability) and negative (0% cell viability) controls, respectively. For LDH assay, PBS and Triton 1% were used as negative (0% LDH release) and positive (100% LDH release) controls, respectively, and results were normalized to 0% and 100% LDH-release. All measurements were expressed as mean ± S.D. ($n = 8$).

3.2. Effects of HePC on passive permeation

3.2.1. Effects of HePC treatment on mannitol and testosterone transport and on TEER

After the determination of subtoxic HePC concentrations, the effects of HePC on the two passive pathways, paracellular and transcellular transport across Caco-2 cell monolayers were assessed.

The apical side of Caco-2 cell monolayers was exposed to various concentrations of HePC for 2 h, from 0 to 100 μM. The effect of HePC exposure on paracellular permeability of the Caco-2 cell monolayers was estimated by the permeability of mannitol—a hydrophilic molecule, which is absorbed exclusively by passive diffusion through the paracellular route [21] and by TEER measurement, while the effect on transcellular permeability was estimated by the permeability of testosterone. These results are shown in Fig. 2. Apical exposure of the monolayers to HePC induced both an increase of mannitol permeability and a decrease of TEER in a concentration-dependent manner. The permeability coefficient for mannitol of untreated cells was $1.1 \pm 0.2 \times 10^{-6}$ cm/s and was in accordance with the literature values [22,23]. After an incubation of Caco-2 monolayers with HePC at a concentration of 100 μM for 2 h, the apparent permeability coefficient P_{app} of mannitol was approximately 20-fold higher and TEER value was reduced to 50%, compared with control. No effect on either absorption of mannitol or TEER were observed after exposure to HePC concentrations lower than 5 μM (even after a 5-h exposure, data not shown). These results showed that HePC clearly enhanced the permeability of the paracellularly transported marker mannitol across intestinal Caco-2 cell monolayers.

Furthermore, Fig. 2 shows that HePC, at the concentrations and exposure times studied, did not affect the permeability of testosterone, indicating that HePC had no effect on the passive transcellular pathway across Caco-2 cell monolayers. The permeability coefficient of testosterone across monolayers of untreated cells was $29.0 \pm 1.3 \times 10^{-6}$ cm/s and was in accordance with the literature values [23]. This result indicated that the permeability increase induced by HePC allowed small molecules such as mannitol to cross the paracellular space whereas the monolayer was still able to provide a barrier function as effective as that of control monolayers against the passage of lipophilic molecules such as testosterone.

3.2.2. Reversibility of HePC-induced effects

In order to determine whether this enhancement of paracellular permeability was deleterious or not for the intestinal barrier, we decided to evaluate the ability of Caco-2 cell monolayers to recover their integrity after exposure to HePC. Monolayers were exposed to 20 μM HePC for 2 h to mimic physiological conditions after an oral administration of the drug. After exposure, monolayers were washed, replaced in

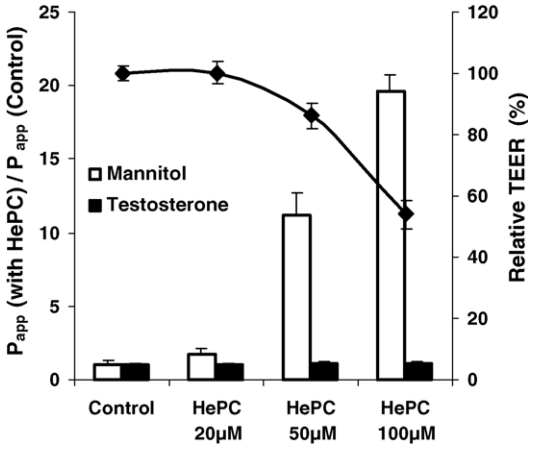

Fig. 2 – Effect of HePC (20, 50 and 100 μM) on TEER and changes in permeability of mannitol as a marker of paracellular transport and testosterone as a marker of transcellular transport across monolayers. HePC was co-administered with [^{14}C]mannitol (0.5 μCi per well) or [^{3}H]testosterone (100 μM, 0.5 μCi per well) on the apical side of Caco-2 cell monolayers and the apparent permeability coefficients (P_{app}) for mannitol and testosterone from apical to basolateral compartment were calculated, as described in Section 2, after 2 h of incubation. Results shown are the ratio of P_{app} calculated from HePC-treated cells/P_{app} calculated from untreated cells (control). TEER was expressed as a percentage of TEER at t_0. The cell viability (>95%) was not compromised, as determined by the MTT assay, at the end of the experiment. All measurements were expressed as mean ± S.D. (n = 9).

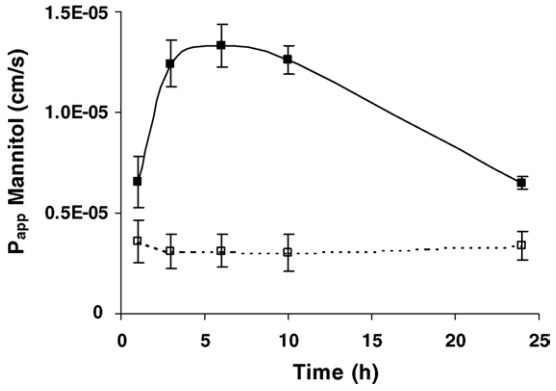

Fig. 3 – Reversibility of effect of HePC on mannitol permeability across Caco-2 cell monolayers. Cells were preincubated for 2 h with HePC 20 μM in the apical compartment (■) or Krebs modified buffer pH 6.0 as a control (□). Cells were then washed three times with fresh buffer and [^{14}C]mannitol (0.1 μCi per well) was added to the apical compartment at t_0. P_{app} for mannitol from apical to basolateral compartment was calculated as described in Section 2, over 24 h. Monolayers were kept at 37 °C and 5% CO_2 during all experiments. All measurements were expressed as mean ± S.D. (n = 9).

fresh medium and the permeability of mannitol was studied over 24 h. Fig. 3 shows the evolution of apparent permeability coefficient. The mannitol P_{app} was two-fold higher than that of untreated control cells after 1 h of recovery and more than four-fold higher after 3 h. The P_{app} of mannitol of treated monolayers reached a peak 6 h after exposure to HePC with a value approximately four-fold higher than that of untreated monolayers, suggesting the existence of a delay before the maximal effect of HePC. Thereafter, paracellular permeability decreased and approched the value for control monolayers. Over the 24-h period of these experiments, cell monolayers did not totally recover paracellular integrity, as indicated by the mannitol permeability value at 24 h, which was two-fold higher than control monolayers. Continuing the experiment for more than 24 h led to a deterioration of untreated monolayer integrity as indicated by the TEER measurement (data not shown). These results demonstrated that recovery of the monolayers after HePC exposure was quite slow but possible and would authorise the co-administration of drugs which undergo passive paracellular transport.

3.2.3. *Influence of the localization of HePC and temperature on HePC-induced effects*

The above results show that HePC increased mannitol permeability when applied apically. To investigate whether this effect was mediated by apical or basolateral stimulation and to clarify the mechanism, a series of experiments was performed in which media containing various concentrations of HePC were added to the apical or basolateral sides of the cells. The results are presented in Fig. 4A. The effects of HePC on the cell monolayers were found to be asymmetrical: the enhancement of mannitol permeability was much more pronounced when HePC was applied to the apical side than when it was added to the basolateral side of Caco-2 cell monolayers. This differential effect does not seem to be due to the polyester membrane, as the transport of HePC from AP to BL compartment was similar to the BL to AP transport (data not shown). Therefore, the apical membrane might be more sensitive. The HePC-induced increase of mannitol permeability (and decrease in TEER, data not shown) was also observed at 4 °C but the effect was less pronounced (Fig. 4B).

3.2.4. *Effects of HePC on tight-junction-associated proteins distribution and on actin filament organization*

The tight junction is a complex structure composed of both transmembrane and cytosolic proteins, which provides a continuous seal around the apical region of the lateral membranes of adjoining epithelial cells, preventing the free passage of molecules by the paracellular pathway. To clarify the mechanism of paracellular permeability enhancement observed after HePC treatment, we investigated its effect on the distribution of four major tight junction-associated proteins in Caco-2 cells, namely ZO-1 (Fig. 5A), occludin (Fig. 5B), E-cadherin (Fig. 5C) and claudin-1 (Fig. 5D). The expression and localization of these proteins were studied by immunostaining followed by confocal laser scanning microscopy (Fig. 5). Control cells showed an uniform distribution of

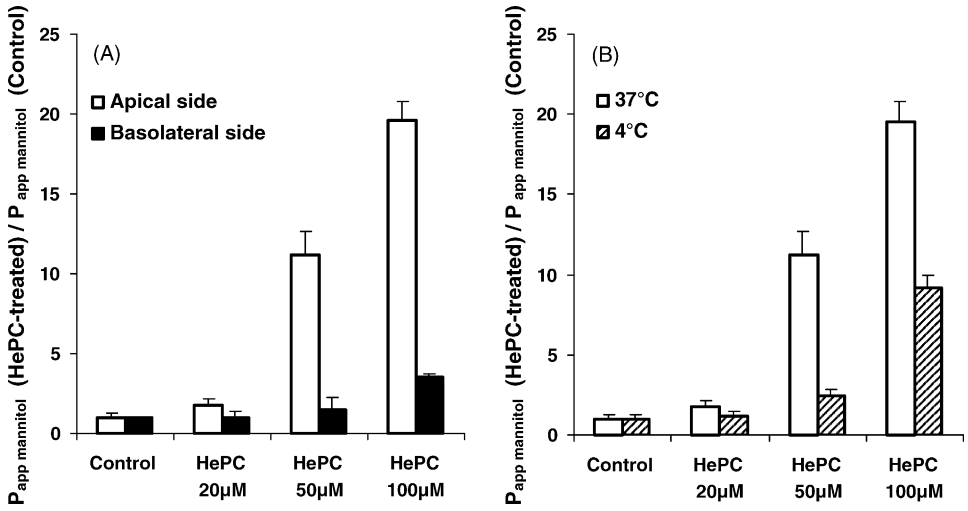

Fig. 4 – Influence of localization of HePC (A) and temperature (B) on mannitol permeability. (A) Monolayers were treated on the apical or basolateral side with HePC at 37 °C, 5% CO_2 and 95% relative humidity. (B) Monolayers were treated apically with HePC at 37 °C or 4 °C. [^{14}C]mannitol (0.5 μCi per well) was added in the apical compartment and the apparent permeability coefficient (P_{app}) from apical to basolateral compartment was calculated as described in Section 2, after 2 h incubation with HePC. The results shown are the ratio of P_{app} calculated from HePC-treated cells/P_{app} calculated from untreated cells (control). All measurements were expressed as mean ± S.D. (n = 9).

the four proteins at intercellular junctions. Monolayers of cells treated with HePC had an abnormal localization and cellular distribution of claudin-1. Its localization within the membrane was not as well defined as that shown by control cells, with a granular appearance and significant presence in the cytosol. The distribution of the other three proteins studied was not affected.

The effect of HePC on F-actin filament organization was also investigated because these filaments are intimately associated with the junctional complex [24]. Staining of F-actin with rhodamine–phalloidin showed that HePC induced changes in the cellular actin distribution (Fig. 6). In many cells, the intensity of the actin staining was reduced and the perijunctional ring of actin filaments was partially disbanded. This effect appeared to be concentrated in the basal region of Caco-2 cells, since the basal actin on the cell border was more discontinuous than in untreated cell monolayers. This result showed that HePC had clear effects on the cytoskeleton and suggests that HePC might cause changes in the interactions of the cytoskeleton with the tight-junction-associated protein claudin-1.

3.3. *Effects of HePC on active transport*

3.3.1. *Effect of a single exposure to HePC on Caco-2 transporter activity*

Since HePC is predominantly incorporated in the cellular lipid compartments, we decided to investigate possible interactions of HePC with physiological intestinal barrier functions, in particular active transport. Three apical membrane proteins transporting very different substrates were studied: (i) the monocarboxylic acid transporter (MCT-1) which can transport a wide range of short-chain monocarboxylates, (ii) the human intestinal peptide transporter (PepT-1) which is responsible for the absorption of di and tripeptides and peptidomimetic drugs such as β-lactam antibiotics in the small intestine, and (iii) the multidrug resistance (MDR1) protein P-glycoprotein (P-gp), which is widely studied as example of an active transport mechanism which expels drugs from the cells, decreasing the intracellular concentration of cytotoxic agents and causing multidrug resistance. These three transporters use energy derived from ATP by direct hydrolysis for P-gp or from a transmembrane proton gradient maintained by a Na^+/H^+ pump for PepT-1 and MCT-1.

To study the effect of a single exposure to HePC, cells were preincubated for 1 h with 20 μM HePC—conditions which were non-toxic and without effect on passive permeation. The functionality of MCT-1, PepT-1 and P-gp in presence or absence of HePC was then evaluated by studying the transport of a known specific substrate of each transporter: butyrate [25], glycylsarcosine (Gly-Sar) [26] and digoxin [27], as previously described. Fig. 7 shows the relative apical to basolateral (AP-BL) permeability coefficients of [^{14}C]butyrate (20 μM), [^{14}C]Gly-Sar (10 μM) and [^{3}H]digoxin (5 μM) in the presence of HePC compared with control. HePC treatment did not significatively affect Gly-Sar or butyrate permeability across Caco-2 cell monolayers indicating that, at the time and dose studied, HePC modified neither PepT-1 nor MCT-1 activity. As far as P-glycoprotein activity was concerned, Fig. 7 clearly shows that HePC increased apical to basolateral permeability of the P-gp substrate digoxin two-fold from 4.05 to 8.05×10^{-6} cm/s ($p < 0.01$). Since the normal activity is the efflux of compounds from the cell at the apical pole, causing a net decrease in AP to BL transport, this result suggested that HePC could inhibit P-gp. A positive control with cyclosporine A—a known P-gp inhibitor—was performed and showed an increase of AP to BL permeability of digoxin, as expected (data not shown).

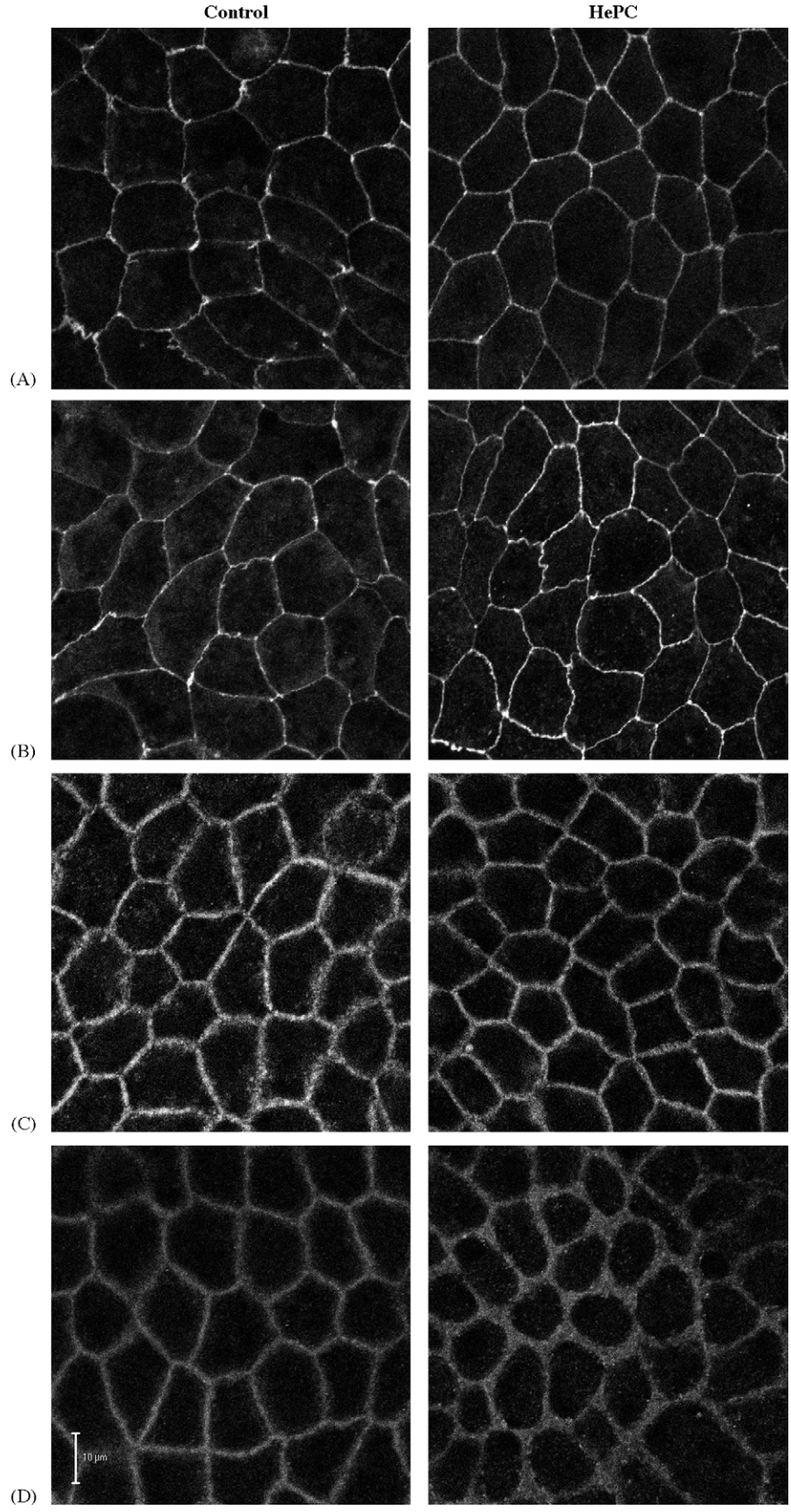

Fig. 5 – Effect of HePC treatment on the distribution of four tight junction-associated proteins in Caco-2 cells, namely ZO-1 (A), occludin (B), E-cadherin (C) and claudin-1 (D). Untreated cells or those treated with 50 μM HePC for 2 h at 37 °C, 5% CO_2 and 95% relative humidity were treated sequentially with primary antibody and FITC-labeled anti-rabbit or anti-mouse IgG antibody and examined with a Zeiss fluorescence confocal microscope. Scale bar 10 μm.

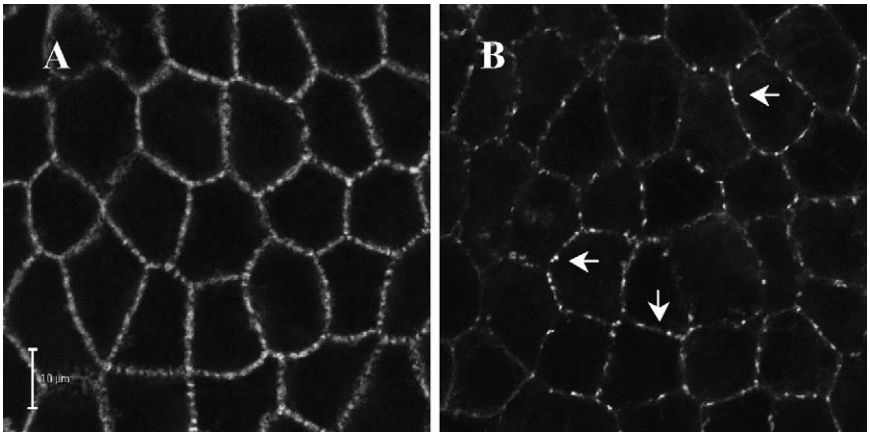

Fig. 6 – Effect of HePC treatment on actin filament organization in Caco-2 cell monolayers. (A) untreated cells and (B) cells treated apically with HePC 50 μM for 2 h at 37 °C, 5% CO_2 and 95% relative humidity. Cell monolayers were then incubated with phalloidin conjugated with rhodamine for 30 min and subsequently washed with PBS, as described in Section 2. Actin filament organization was viewed with a Zeiss fluorescence confocal microscope. Scale bar 10 μm. The arrows indicate regions of actin redistribution in the basal region.

3.3.2. Effect of chronic exposure to HePC on P-gp expression

Fig. 7 suggested an interaction between HePC and the P-glycoprotein in the Caco-2 cell line. Since VL treatment consists in daily oral administration of HePC for 28 days, we exposed the apical side of Caco-2 cells to HePC for 4 weeks in order to mimic physiological conditions and thereby evaluate whether a chronic exposure of the intestinal barrier with HePC could modify the P-gp activity. The expression of P-gp in control cells and in HePC-treated cells determined by Western blot analysis is presented in Fig. 8. This shows that chronic exposure of the cells to HePC did not lead to a decrease of expression of the protein, as revealed by the densitometric analysis of immunoblots. Thus, oral administration of HePC for 28 days as described [28] would not be expected to induce P-gp modulation.

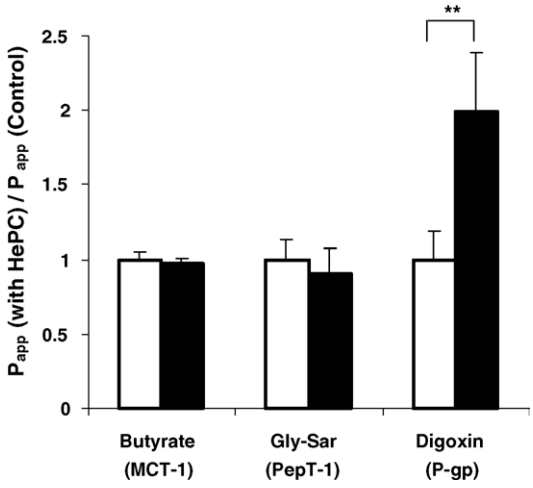

Fig. 7 – Effects of HePC on MCT-1, PepT-1 and P-gp activities. Caco-2 monolayers were preincubated with HePC 20 μM (■) or Krebs buffer as control (□) for 1 h. HePC (20 μM) was co-administrated with 0.5 μCi per well of [^{14}C]butyrate (20 μM), [^{14}C]Gly-Sar (10 μM) or [^{3}H]digoxin (5 μM) on apical side and P_{app} from apical to basolateral compartment was calculated as described in Section 2. The results shown are the ratio of P_{app} calculated from cell treated with HePC/P_{app} calculated from untreated cells (control). Mean ± S.D. (n = 9) $^{**}p < 0.01$ vs. control.

4. Discussion

Little is known about the impact of oral administration of HePC on the intestinal barrier despite the physiological and therapeutic interest of this molecule. We therefore used Caco-2 cell monolayers to study the dose-dependent effects on the intestinal epithelium of short- and long-term exposure to HePC and its influence on the three major routes of transepithelial movement namely: (1) transcellular passive diffusion, (2) paracellular passive diffusion and (3) carrier-mediated transcellular transport.

We demonstrated that HePC had no effect on testosterone permeability but increased mannitol permeability and decreased TEER values without irreversible cell damage. These effects were time, dose and temperature-dependent and could not be ascribed to aspecific cytotoxicity – under our experimental conditions – as shown by cell viability controls by MTT and LDH measurement. Taken together, these results clearly indicate that HePC enhances passive paracellular permeability across Caco-2 cell monolayers without affecting the passive transcellular route and are in agreement with previous studies conducted with similar compounds in this [7–9] and other epithelial cell lines [6].

Permanent disruption of the barrier properties after treatment of the intestinal epithelium could lead to problems such as an increased risk of infection of the area. Therefore the ability of Caco-2 cells to recover from treatment with HePC was investigated. The permeability of mannitol across HePC-treated monolayers approched control values after 24 h of recovery but complete recovery was not observed. This seems in contrast with previous studies with dodecylphosphocholine

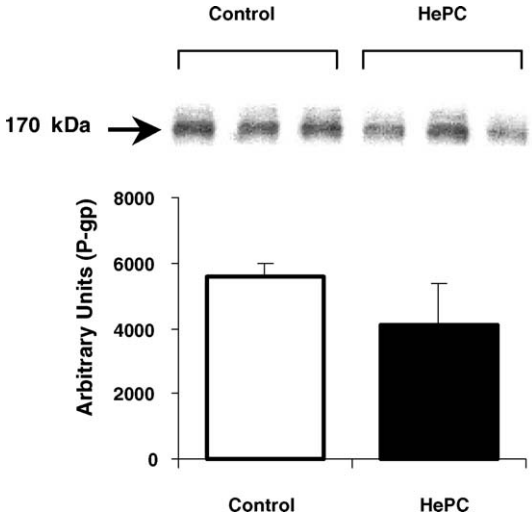

Fig. 8 – Effect of chronic treatment with HePC on P-glycoprotein expression in Caco-2 cells. Caco-2 cells were cultivated for 3 weeks and then seeded into 6-well plates and grown for 18 days. The medium was supplemented with HePC 5 μM throughout this period. P-gp expression was then evaluated by Western blot analysis in 20 μg of total proteins using the mouse monoclonal C219 antibody. One immunoreactive protein of 170 kDa was detected. Densitometric analysis of immunoblots was performed using the NIH image analysis (Scion Image Corporation). Data are expressed in arbitrary units as mean ± S.D. of $n = 3$ wells per group. The difference was not statistically significant ($p > 0.05$).

(DPC) [7] in which DPC-treated monolayers recovered full integrity after 10 h in fresh medium. This difference may be due to the different nature of the phospholipids themselves but also to the duration of treatment, since Lui et al. performed only 30 min of treatment with DPC. Our study also showed that a latent period was necessary before the full effect of HePC was evident. Indeed, even after removal of the drug in contact with the cells, the mannitol permeability across Caco-2 monolayers kept increasing. This latent period and the slow recovery observed in our experiments could also be explained by the hypothesis of HePC molecules sequestered within the cell membranes, influencing paracellular permeability although the drug was not still present in the medium.

The permeability increase induced by HePC could allow small molecules to cross the intestinal barrier via the paracellular space and could therefore be exploited to improve the transport of other therapeutic agents co-administered with HePC. An example of co-administration could be bitherapy for visceral leishmaniasis. Although HePC is extremely active against *Leishmania donovani*, the ease with which resistant mutants can be produced in the laboratory is a cause of concern [29]. Thus, to reduce and postpone the emergence of resistant mutants, it might be advantageous to use HePC in combination with other anti-leishmanial drugs. Antiparasitic drugs such as amphotericin B, atovaquone and artemisinin, which are poorly soluble in water, are hardly absorbed via the gastrointestinal tract (GIT) and exhibit only low oral bioavailability. Co-administration with HePC could promote their paracellular transport by reversibly opening epithelial tight junctions and therefore improve their oral availability.

HePC is a phospholipid analogue and it is important to note that our results can be compared to previous studies on the effects of lipid surfactants on epithelial barriers. Although the mechanism is still unclear, it has been shown that these surfactants can increase the permeability of epithelial barriers in a concentration-dependent manner [12]. Furthermore, it has been suggested that phospholipid derivatives increase the paracellular permeability of hydrophilic compounds by modulation of tight junctions [7,13,14]. Here we provide information that HePC can act in the same way in Caco-2 cell monolayers.

There are several possible mechanisms by which HePC could affect the integrity of the tight junctions. The hypothesis that the protein complex is disrupted by solubilization of the lipid membrane can be discounted since it has recently been demonstrated that HePC is not able to solubilize the membrane lipids [30]. Our experiments also demonstrated that the effect of HePC on paracellular permeability was reduced but not abolished at 4 °C, suggesting that HePC did not disturb the tight junctional complex by an ATP-dependent pathway. Furthermore, the asymmetrical effect of HePC on paracellular permeability depending on whether it was applied to the apical or basolateral pole could be explained by several hypotheses: (i) a polarized activity of HePC depending on membrane phospholipid composition, since it is known that apical and basal membranes differ in lipid composition; (ii) a carrier-mediated effect of HePC with receptors located the apical membrane of Caco-2 cells; (iii) a membrane phospholipid composition-dependent uptake of HePC by Caco-2 cells. Indeed, it is known that HePC has multiple effects on cell membranes [31] and membrane composition may be an important factor in the enhancement of paracellular transport.

To investigate the effect of HePC on paracellular permeability further, its influence on tight junction proteins and on actin organization were examined at a concentration which caused a decrease in TEER and an increase in paracellular permeability. In the intestinal Caco-2 cell model, HePC treatment appeared to alter the distribution of one of the four tight junction-associated proteins studied, claudin-1, a transmembrane protein, and provoked a dramatic disorganization of cytoskeletal actin filaments. Indeed, immunofluorescent analysis of claudin-1 expression showed an abnormal distribution of the protein in cells treated with HePC compared with the well-defined localization of claudin-1 in membranes of control cells. Furthermore, there were granules of claudin-1 present in the cytosol of HePC-treated cells. Thus, for the first time we demonstrate that claudin-1 localization may be involved in the enhancement of passive paracellular permeability induced by HePC in Caco-2 cells. This result is consistent with the fact that claudins are the major structural components of tight junctional strands [12]. Therefore, a modulation of this crucial protein for the assembly of intracellular tight junction by HePC would lead to an increase of epithelial permeability. On the other hand, no redistribution of the tight junction-associated protein ZO-1 was observed in

our experiments, in contrast to previous studies which reported changes in ZO-1 protein distribution after ET-18-OCH_3 treatment on T84 cells [5] and with dodecylphosphocholine on Caco-2 monolayers [7]. This suggests that the effect of HePC is not exactly identical to other ALPs and may be cell line-dependent.

Recently, it has been demonstrated that phospholipase C-β (PLC-β) modulates tight junction permeability by affecting actin filament organization and that PLC-β inhibition leads to increased tight junction permeability across MDCK cell monolayers [6]. Since HePC has been shown to inhibit PLC-β [32], we suggest that HePC is able to increase tight junction permeability in Caco-2 cell monolayers by PLC-β-mediated reorganization of the actin ring.

In conclusion, we have demonstrated here that the interaction of HePC, used at subtoxic doses, with the Caco-2 cell membrane results in a structural reorganization of the tight junction-associated protein claudin-1 and a disruption in actin filament organization which is followed by enhanced transport through the passive paracellular pathway. In this way HePC could compromise the barrier function of the Caco-2 cell monolayers. Further research is required to examine the full nature and molecular basis of these effects of HePC on the intestinal barrier.

In this study, we also decided to elucidate the impact of oral administration of HePC on carrier-mediated transport across the intestinal barrier. The three transporters chosen are capable of transporting a large number of pharmacological agents and any modification of their transport capacities could have major repercussions on the bioavailability of these drugs. Indeed, PepT-1 is involved in the absorption of di and tripeptides, arising from the digestion of dietary proteins in the small intestine but also has a broad substrate specificity and is capable of transporting several compounds, which possess structural features similar to those of small peptides, for example β-lactam antibiotics, angiotensin converting enzyme (ACE) inhibitors, prodrugs of acyclovir, gancyclovir, L-dopa and pamidronate. MCT-1, the proton-linked monocarboxylate transporter, is involved in the transport of a wide range of short chain monocarboxylic acids such as acetic acid and nicotic acid. Finally, P-glycoprotein, a membrane ATPase extensively expressed in the intestinal barrier, is involved in the outward transport of several cytotoxic agents and contributes to reduced oral absorption of drugs. Previous studies have reported that several non-ionic surfactants (i.e. Tween 80 and Cremophor EL) with membrane fluidity modulating activity were able to inhibit P-gp and the peptide transporter in a concentration-dependent manner [16]. Interestingly, in our experiments, HePC treatment of Caco-2 cells did not alter PepT-1 or MCT-1 activities, but caused an inhibition of the intestinal P-glycoprotein activity. These results suggest differences of sensitivity of these cellular transport systems towards HePC and showed that HePC did not simply influence membrane transporter function by modification of membrane fluidity or lipid organization but strongly suggest that HePC could inhibit P-gp in a more specific way. This observed inhibition of P-gp is consistent with the fact that protein kinase C (PKC) blockers inhibit P-gp-mediated drug transport [33], since HePC is known to be a potent inhibitor of PKC [34,35]. The mechanism and concentration dependence of HePC-induced P-gp inhibition in Caco-2 cells remains to be investigated.

This observed HePC-induced P-gp inhibition could have major repercussions on the use of HePC in leishmaniasis treatment. Indeed, oral HePC has proven to be highly effective against visceral leishmaniasis in patients with human immunodeficiency virus (HIV)-*Leishmania* coinfection [36], a serious new disease pattern, which is becoming increasingly frequent. However, the interactions between antiretrovirals and anti-leishmanials (especially HePC) have not been studied. Protease inhibitors such as indinavir, saquinavir, ritonavir, nelfinavir and non-nucleoside reverse-transcriptase inhibitors such as efavirenz used in HIV treatment are known to be P-gp substrates [37,38]. Therefore, our present results suggest that HePC, by virtue of its inhibition of the intestinal P-gp, could have the potential to enhance gastrointestinal tract absorption of P-gp substrates by decreasing P-gp-mediated drug efflux.

Since we had demonstrated that short-term exposure of HePC on epithelial Caco-2 cells leads to an inhibition of the P-glycoprotein, we decided to evaluate the consequence of a long-term exposure. Our results show that chronic exposure of intestinal cells to low doses of HePC did not modify the level of P-gp expression, suggesting that the observed influence of short-term exposure HePC on P-gp should be attributed to a direct interaction with the protein. The molecular interaction between HePC and P-gp remains to be investigated.

In summary, we demonstrate that: (i) HePC had no influence on the passive transcellular pathway, (ii) HePC enhanced paracellular permeation across Caco-2 cell monolayers, (iii) HePC reversibly opened Caco-2 epithelial tight junctions via disorganization of claudin-1 and disruption of actin filaments, (iv) HePC single short-term exposure led to an inhibition of intestinal P-gp activity. These observations open new perspectives for using HePC to improve the transport of other therapeutic molecules by the oral route.

Acknowledgement

The authors would like to thank Valerie Nicolas (Service Commun d'Imagerie, Institut Fédératif de Recherche INSERM 75) for valuable help in operating the confocal microscope.

REFERENCES

[1] Sundar S, Makharia A, More DK, Agrawal G, Voss A, Fischer C, et al. Short-course of oral miltefosine for treatment of visceral leishmaniasis. Clin Infect Dis 2000;31:1110–3.
[2] Guerin P, Olliaro P, Sundar S, Boelaert M, Croft S. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. Lancet 2002;2:494–501.
[3] Van Blitterswijk WJ, Hilkmann H, Storme GA. Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. Lipids 1987;22:820–3.
[4] Berkovic D, Grunwald U, Menzel W, Unger C, Hiddemann W, Fleer EA. Effects of hexadecylphosphocholine on membrane phospholipid metabolism in human tumour cells. Eur J Cancer 1995;31A:2080–5.

[5] Leroy A, de Bruyne GK, Oomen LC, Mareel MM. Alkylphospholipids reversibly open epithelial tight junctions. Anticancer Res 2003;23:27–32.
[6] Ward PD, Ouyang H, Thakker DR. Role of phospholipase C-beta in the modulation of epithelial tight junction permeability. J Pharmacol Exp Ther 2003;304:689–98.
[7] Liu DZ, LeCluyse EL, Thakker DR. Dodecylphosphocholine-mediated enhancement of paracellular permeability and cytotoxicity in Caco-2 cell monolayers. J Pharm Sci 1999;88:1161–8.
[8] Liu DZ, Morris-Natschke SL, Kucera LS, Ishaq KS, Thakker DR. Structure–activity relationships for enhancement of paracellular permeability by 2-alkoxy-3-alkylamidopropylphosphocholines across Caco-2 cell monolayers. J Pharm Sci 1999;88:1169–74.
[9] Ouyang H, Morris-Natschke SL, Ishaq KS, Ward P, Liu D, Leonard S, et al. Structure–activity relationship for enhancement of paracellular permeability across Caco-2 cell monolayers by 3-alkylamido-2-alkoxypropylphosphocholines. J Med Chem 2002;45:2857–66.
[10] Hovgaard L, Brondsted H. Drug delivery studies in Caco-2 monolayers IV. Absorption enhancer effects of cyclodextrins. Pharm Res 1995;12:1328–32.
[11] Drejer K, Vaag A, Bech K, Hansen P, Sorensen AR, Mygind N. Intranasal administration of insulin with phospholipid as absorption enhancer: pharmacokinetics in normal subjects. Diabet Med 1992;9:335–40.
[12] Ward PD, Tippin TK, Thakker DR. Enhancing paracellular permeability by modulating epithelial tight junctions. Pharm Sci Technol Today 2000;3:346–58.
[13] Schulze C, Smales C, Rubin LL, Staddon JM. Lysophosphatidic acid increases tight junction permeability in cultured brain endothelial cells. J Neurochem 1997;68:991–1000.
[14] Brondsted H, Nielsen HM, Hovgaard L. Drug delivery studies in Caco-2 monolayers III. Intestinal transport of various vasopressin analogues in the presence of lysophosphatidylcholine. Int J Pharm 1995;114:151–7.
[15] Koga K, Murakami M, Kawashima S. Modification of ceftibuten transport by changes in lipid fluidity caused by fatty acid glycerol esters. Biol Pharm Bull 1999;22:103–6.
[16] Rege BD, Kao JP, Polli JE. Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. Eur J Pharm Sci 2002;16:237–46.
[17] Nerurkar MM, Burton PS, Borchardt RT. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. Pharm Res 1996;13:528–34.
[18] Artursson P. Epithelial transport of drugs in cell culture I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J Pharm Sci 1990;79:476–82.
[19] Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736–49.
[20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[21] Artursson P, Ungell AL, Lofroth JE. Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments. Pharm Res 1993;10:1123–9.
[22] Demirbas S, Stavchansky S. Effects of citicholine and dimethylsulfoxide on transepithelial transport of passively diffused drugs in the Caco-2 cell culture model. Int J Pharm 2003;251:107–12.
[23] Markowska M, Oberle R, Juzwin S, Hsu CP, Gryszkiewicz M, Streeter AJ. Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis. J Pharm Tox Methods 2001;46:51–5.
[24] Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, et al. Tight junctions are membrane microdomains. J Cell Sci 2000;113:1771–81.
[25] Hadjiagapiou C, Schmidt L, Dudeja PK, Layden TJ, Ramaswamy K. Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. Am J Physiol Gastrointest Liver Physiol 2000;279:G775–80.
[26] Thwaites DT, Hirst BH, Simmons NL. Substrate specificity of the di/tripeptide transporter in human intestinal epithelia (Caco-2): identification of substrates that undergo H(+)-coupled absorption. Br J Pharmacol 1994;113:1050–6.
[27] Cavet ME, West M, Simmons NL. Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. Br J Pharmacol 1996;118:1389–96.
[28] Sundar S, Rosenkaimer F, Makharia MK, Goyal AK, Mandal AK, Voss A, et al. Trial of oral miltefosine for visceral leishmaniasis. Lancet 1998;352:1821–3.
[29] Seifert K, Matu S, Javier Perez-Victoria F, Castanys S, Gamarro F, Croft SL. Characterisation of Leishmania donovani promastigotes resistant to hexadecylphosphocholine (miltefosine). Int J Antimicrob Agents 2003;22:380–7.
[30] Rey Gomez-Serranillos I, Minones Jr J, Dynarowicz-Latka P, Minones J, Conde O. Surface behavior of oleoyl palmitoyl phosphatidyl ethanolamine (OPPE) and the characteristics of mixed OPPE-miltefosine monolayers. Langmuir 2004;20:11414–21.
[31] Berkovic D, Wernicke JH, Fleer EA. Effects of etherlipid analogs on cell membrane functions. J Exp Ther Oncol 2003;3:185–93.
[32] Powis G, Seewald MJ, Gratas C, Melder D, Riebow J, Modest EJ. Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. Cancer Res 1992;52:2835–40.
[33] Castro AF, Horton JK, Vanoye CG, Altenberg GA. Mechanism of inhibition of P-glycoprotein-mediated drug transport by protein kinase C blockers. Biochem Pharmacol 1999;58:1723–33.
[34] Berkovic D, Luders S, Goeckenjan M, Hiddemann W, Fleer EA. Differential regulation of phospholipase A2 in human leukemia cells by the etherphospholipid analogue hexadecylphosphocholine. Biochem Pharmacol 1997;53:1725–33.
[35] Uberall F, Oberhuber H, Maly K, Zaknun J, Demuth L, Grunicke HH. Hexadecylphosphocholine inhibits inositol phosphate formation and protein kinase C activity. Cancer Res 1991;51:807–12.
[36] Thakur CP, Sinha PK, Singh RK, Hassan SM, Narain S. Miltefosine in a case of visceral leishmaniasis with HIV co-infection; and rising incidence of this disease in India. Trans R Soc Trop Med Hyg 2000;94:696–7.
[37] Kageyama M, Namiki H, Fukushima H, Ito Y, Shibata N, Takada K. In vivo effects of cyclosporin A and ketoconazole on the pharmacokinetics of representative substrates for P-glycoprotein and cytochrome P450 (CYP) 3A in rats. Biol Pharm Bull 2005;28:316–22.
[38] Veau C, Faivre L, Tardivel S, Soursac M, Banide H, Lacour B, et al. Effect of interleukin-2 on intestinal P-glycoprotein expression and functionality in mice. J Pharmacol Exp Ther 2002;302:742–50.