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*Research Paper*

# Intestinal Absorption of Miltefosine: Contribution of Passive Paracellular Transport

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***Purpose.*** This study aimed to characterize the transepithelial transport of miltefosine (HePC), the first orally effective drug against visceral leishmaniasis, across the intestinal barrier to further understand its oral absorption mechanism.
***Materials and Methods.*** Caco-2 cell monolayers were used as an *in vitro* model of the human intestinal barrier. The roles of active and passive mechanisms in HePC intestinal transport were investigated and the relative contributions of the transcellular and paracellular routes were estimated.
***Results.*** HePC transport was observed to be pH-independent, partially temperature-dependent, linear as a function of time and non-saturable as a function of concentration. The magnitude of HePC transport was quite similar to that of the paracellular marker mannitol, and EDTA treatment led to an increase in HePC transport. Furthermore, HePC transport was found to be similar in the apical-to-basolateral and basolateral-to-apical directions, strongly suggesting that HePC exhibits non-polarized transport and that no MDR-mediated efflux was involved.
***Conclusions.*** These results demonstrate that HePC crosses the intestinal epithelium by a non-specific passive pathway and provide evidence supporting a concentration-dependent paracellular transport mechanism, although some transcellular diffusion cannot be ruled out. Considering that HePC opens epithelial tight junctions, this study shows that HePC may promote its own permeation across the intestinal barrier.

**KEY WORDS:** Caco-2; hexadecylphosphocholine; intestinal absorption; paracellular transport; miltefosine.

## INTRODUCTION

Miltefosine (hexadecylphosphocholine, HePC), an alkylphosphocholine (APC), is the first effective oral agent available for treating visceral leishmaniasis (VL) (1,2) with a 98% cure rate of VL patients with a dose of 2.5 mg/kg daily for 28 days (3). HePC is considered as a major breakthrough in antileishmanial treatment because it can be administered orally in contrast to the currently recommended antileishmanial drugs that require parenteral administration.

There are few published data available on the experimental or clinical pharmacokinetics of HePC. A study on the oral bioavailability in mice demonstrated that HePC is well absorbed from the intestinal tract and that oral administration and intravenous injection lead to a similar distribution throughout the body (4). Another study in the rat also reported the complete absorption of the compound from the gut and its oral bioavailability was found to be 82% (5). Furthermore, *in vivo* studies have demonstrated that HePC was first detected in serum 6 h after oral administration (6). Although data on the good bioavailability and the remarkable activity of HePC after oral administration exist, there is no information on the mechanism(s) of its absorption across the human epithelial intestinal barrier. In fact, very few studies have focused on the transport of HePC and alkyllysophospholipids (ALPs) across biological membranes in general. This question is all the more relevant since, to be active against *Leishmania* parasites, HePC has to cross the intestinal epithelium, the blood vessel endothelium, the host cell plasma membrane, the phagolysosomal membrane and finally the parasite membrane.

The transport of drugs across the intestinal epithelium may occur by one or more of four different routes: the passive transcellular and paracellular routes, the carrier-mediated route and by transcytosis. The main properties determining absorption, distribution and transport of a drug *in vivo* are solubility, lipophilicity, stability, and acid-base character. HePC is a neutral detergent, composed of a

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**ABBREVIATIONS:** AP, apical; APC, alkylphosphocholine; BL, basolateral; HePC, hexadecylphosphocholine (miltefosine); $P_{app}$, apparent permeability coefficient; *P*-gp, *P*-glycoprotein.

zwitterionic headgroup (a negative phosphoryl group and a positive choline) and a C16 hydrocarbon chain (MW 408). The phosphocholine polar group bears both positive and negative charges within the pH range of 2–12 (7), and so has little net charge at the physiological pH of 7.4. This amphiphilic molecule is extremely water-soluble, forming micelles with a critical micellar concentration (CMC) of 8–12 μM (8,9). The solubility of HePC in ethanol, DMSO, dimethyl formamide and PBS (pH 7.2) is 1.25 mg/ml, 800 μg/ml, 50 μg/ml and at least 4 mg/ml, respectively; and its oil–water partition coefficient value is 30 (10) (i.e. Log*P*=1.48, characteristic of a slightly lipophilic compound). Moreover, HePC is the first drug based on a phospholipid-like structure to enter clinical use, and it is known to adsorb to the cell membrane surface and accumulate within the phospholipid bilayer (11,12). All these physicochemical features are compatible with an absorption by passive diffusion. On the other hand, several observations in the literature suggest a possible involvement of an active or carrier-mediated mechanism in HePC transport. Indeed, an HePC transporter has recently been identified and characterized in *Leishmania donovani* parasites (13).

The aim of the present study was to investigate the mechanism of HePC transport across the human intestinal barrier. The human-derived colon carcinoma epithelial cell line Caco-2 grown on Transwell®-clear polyester membranes was used as a validated intestinal transport model system (14,15). The objectives of this study were: (1) to determine whether the HePC transport across the intestinal barrier involved an active or a passive mechanism and (2) to evaluate the transcellular and paracellular pathway involved in HePC transport.

## MATERIALS AND METHODS

### 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) and Ultima Gold™ liquid scintillation were from Perkin Elmer Life Science Products (Boston, USA). [9,10-$^{3}$H]HePC was synthesized as described previously (16,17). The purity of the [$^{3}$HC$^{9}$-$^{3}$HC$^{10}$]HePC synthesized was monitored by β-imaging and was found to be 96%. Working solutions were prepared with isotopic dilution and the specific activity was fixed at 50 μCi/mmol. HePC was obtained from Cayman Chemical Company (Ann Arbor, USA, Michigan). 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, 100X), penicillin–streptomycin solution (10,000 units/ml penicillin and 10 mg/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) and 12-well cluster trays were purchased from the Costar Corning Corporation (New York, USA). Testosterone was from Fluka Chemika (Buchs, Switzerland). All other chemical products were from Sigma-Aldrich (St Louis, MO, USA).

### Cell Culture

The human cell line Caco-2 was established from a moderately well-differentiated colon adenocarcinoma. This cell line was a gift from A. Bado (INSERM U773, UFR de Médecine Paris 7—Denis Diderot). 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. All cultures were mycoplasma-free. Every week, cells were trypsinized and seeded at $5\times10^4$ cells per insert onto Transwells® 12-well for transport studies and at $5\times10^4$ cells per 12-well cluster trays 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 [$^{14}$C]mannitol and the transepithelial electrical resistance (TEER) using an EVOM Epithelial Tissue Voltohmmeter (World Precision Instrument, Sarasota, FL). Monolayers that displayed a TEER of 200–300 Ω cm$^2$ were used in the experiments. For all the experimental conditions used, cell viability and plasma membrane integrity were checked using the MTT colorimetric assay (18) and the LDH release assay (19), respectively, as previously described (20).

### Transepithelial Transport

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 culture medium of apical and basolateral compartments was removed by aspiration and the 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 the radiolabeled compound ([$^{3}$H]HePC, [$^{14}$C]mannitol, [$^{3}$H]testosterone) was added to the apical (0.5 ml) or basolateral (1.5 ml) compartment of the insert and the flux across Caco-2 cell monolayers was measured under various conditions. 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 the cell monolayer was determined. For this, monolayers were washed three times with ice-cold buffer, solubilized with Triton-X100 at 1% and processed for scintillation counting.

The apparent permeability coefficient $P_{app}$ (cm/s) for each of the radiolabeled compounds was 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.

### Transport After Energy Depletion

To investigate whether the HePC transport was energy-dependent, total intracellular ATP level was reduced using a modification of a method previously described (21). Both the oxidative phosphorylation and the glycolytic pathways were blocked by omitting glucose from the transport medium and supplementing it with sodium azide ($NaN_3$, 10 mM), sodium fluoride (NaF, 2 mM) and 2-deoxyglucose (2-DG, 50 mM) in both compartments. After a 30-min incubation at 37°C, medium was removed, [$^3$H]HePC (20 μM, 0.5 μCi per well) was added to the apical compartment and the $P_{app}$ was determined as described above.

### Transport After Alteration of the Tight Junction Barrier

EDTA is known to modulate the paracellular permeability by altering tight junctions (22). Therefore to compare the transport rate of mannitol—a paracellular marker—and HePC without the functional barrier of epithelial tight junctions, EDTA was used to open the tight junctions completely. A 10 μM solution of EDTA was prepared using PBS without $Ca^{2+}$/$Mg^{2+}$. The solution was applied to the apical and basolateral sides of Caco-2 cell monolayers for 5 min at 37°C. The solution was removed and the cells were washed three times with PBS without $Ca^{2+}$/$Mg^{2+}$. Then [$^{14}$C]mannitol (0.5 μCi per well), [$^3$H]testosterone (100 μM, 0.5 μCi per well) or [$^3$H]HePC (20 μM, 0.5 μCi per well) was added to the apical compartment and the $P_{app}$ was determined as described previously.

### Release of HePC from Cells

For measurement of HePC release from the cells, Caco-2 monolayers grown on permeable supports were preincubated with [$^3$H]HePC (20 μM, 0.5 μCi per well) in Krebs modified buffer added to the apical compartment for 2 h at 37°C. Thereafter, apical and basolateral buffer were removed, the cells were washed three times and efflux of HePC from the basolateral side of the monolayer was monitored at 37°C for different times up to 24 h.

### Statistics

All experiments were conducted at least in triplicate and 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 or a Student's *t*-test (GraphPad Instat, San Diego, CA). Statistical significance was accepted as $p<0.05$.

## RESULTS

### pH-dependence and the Effect of Albumin

The effect of pH on the transepithelial transport of 20 μM [$^3$H]HePC from the apical (AP) to the basolateral (BL) side of Caco-2 cell monolayers was investigated. The pH on the apical side was changed from 6.0 to 7.4, while the basolateral pH was fixed at pH 7.4, corresponding to the physiological luminal pH. Figure 1 shows that the $P_{app}$ of HePC across the Caco-2 cell monolayers did not change significantly with a decrease of pH on the apical side, indicating that HePC transport across the intestinal barrier is proton gradient-independent. The $P_{app}$ values were 2.69±0.61 and 2.68±0.69×$10^{-6}$ cm/s for an apical pH of 7.4 and 6.0, respectively. In subsequent studies, transport was studied at the apical pH of 6.0 and the basolateral pH of 7.4 in order to better mimic physiological conditions.

Furthermore, the influence of adding human serum albumin to the basal compartment was studied. As shown in Fig. 1, the AP-to-BL permeability coefficient of HePC increased markedly ($p<0.01$), whereas the BL-to-AP permeability was significantly reduced (data not shown).

### Time-dependence and Bidirectional Transport of HePC

The time course of [$^3$H]HePC transport across Caco-2 cell monolayers was studied at 37°C in both the AP-to-BL and the BL-to-AP directions (Fig. 2). The amount of HePC crossing the monolayer increased linearly with respect to time from 0 to 3 h with no latent period, and was therefore non-saturable over the time period studied. Furthermore, transport profiles of HePC across Caco-2 monolayers as a function of time were independent of the direction and the apparent permeability coefficients $P_{app}$ did not differ significantly. These were 2.68±0.69 and 2.72±0.13×$10^{-6}$ cm/s for the AP-to-BL (absorptive) and for the BL-to-AP (secretory) direction respectively.

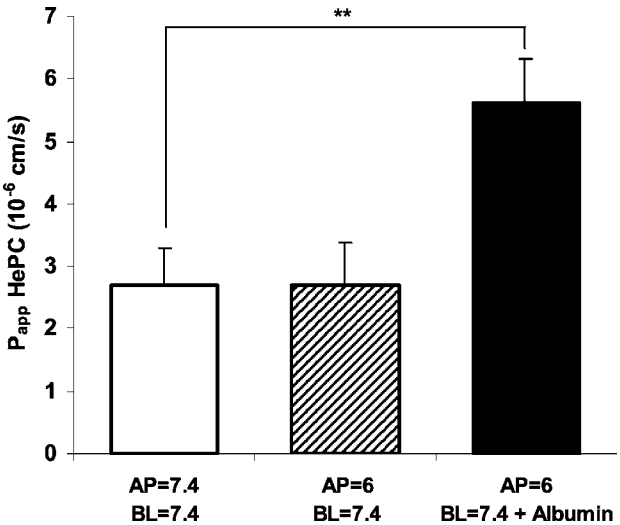


**Fig. 1.** Effect of pH and human serum albumin on transepithelial transport of HePC in Caco-2 cells monolayers. Apical-to-basolateral (*AP-to-BL*) transport of [$^3$H]HePC (20 μM, 0.5 μCi per well) was measured under different conditions: AP and BL pH of 7.4, AP pH of 6.0 and BL pH of 7.4 or AP pH of 6.0 and a BL pH of 7.4 in the presence of 5 mg/ml human serum albumin in the BL compartment. The monolayers were incubated at 37°C for 3 h and the apparent permeability coefficient ($P_{app}$) from AP-to-BL was calculated as described in the "Materials and Methods" section. Results are the mean±S.D. of three determinations with three different monolayers ($n=9$). **$p<0.01$.

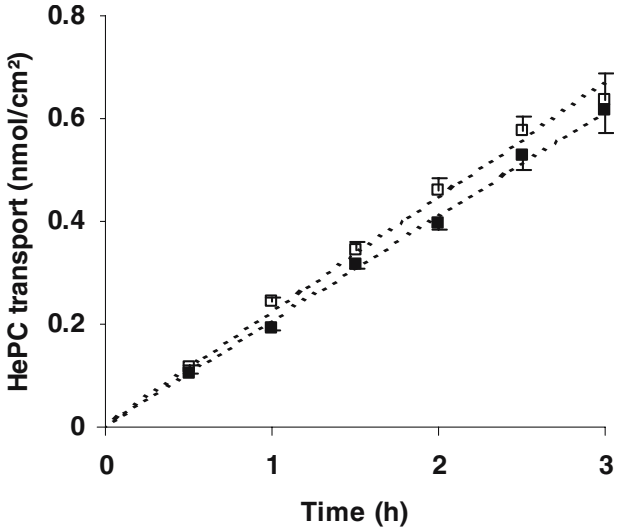


**Fig. 2.** Time course of transepithelial transport of [$^3$H]HePC (20 μM, 0.5 μCi per well) from the apical to the basolateral (*empty square*) and from the basolateral to the apical (*filled square*) side of Caco-2 cell monolayers. The transport was characterized by linear regression analysis ($R^2$>0.98 in both cases). All measurements are expressed as mean±S.D. of three determinations with three different monolayers ($n$=9).

At the end of this transepithelial transport experiment, the distribution of HePC was calculated by determining the intracellular accumulation of HePC and the total accumulation in the basolateral compartment. Interestingly, accumulation of HePC within Caco-2 cells was very high compared with transport across the monolayer. Indeed, after a 3-h incubation on the apical side, the drug incorporated or sequestered within the cells was found to be more than 56.8±2.2% of HePC added at the beginning of the experiment ($n$=12), corresponding to 6.00±0.13 nmol/monolayer/3 h (for 20 μM HePC added); whereas the total amount of HePC transported across the epithelium was found to be 6.8±0.4% of the dose initially added to the apical compartment. However, this result confirmed a significant transport of HePC across the intestinal epithelium.

## HePC Release from Caco-2 Cell Monolayers

Since a high proportion of added HePC was found within the cells, the possibility of subsequent HePC efflux from Caco-2 cells was investigated. Therefore, cells were preincubated for 2 h with [$^3$H]HePC at the apical side, washed and then incubated in fresh transport buffer for different times. The amounts of HePC released from the cells at the basolateral pole as a function of time are depicted in Fig. 3. This HePC efflux was found to be saturable. However, after 24 h, only 5% of the incorporated HePC was released to the basolateral compartment, corresponding to 0.10±0.01 nmol. This revealed a very slow efflux of HePC, indicating that the drug was partially excreted by Caco-2 cells but was preferentially sequestered within the cells. It has to be noted that the saturable efflux of HePC could not be ascribed to HePC solubility, as sink conditions were maintained throughout the experiment.

## Temperature and Energy-dependence of HePC Transport

The effect of temperature on the transepithelial transport of HePC was determined by measuring the $P_{app}$ at 37 and 4°C, in order to determine the possible involvement of an energy-dependent mechanism in HePC transport across Caco-2 cell monolayers. Table IA shows that, at 4°C, the transport of HePC was not abolished but was reduced 2-fold compared with the transport at 37°C. The effect of a decrease of experimental temperature on the permeability of Caco-2 monolayers was further investigated by studying the $P_{app}$ of mannitol and testosterone, known markers of paracellular and transcellular passive permeability, respectively. The $P_{app}$ of HePC was then compared with the $P_{app}$ values of these model compounds. The results showed that $P_{app}$ of mannitol and testosterone were also reduced two-fold at 4°C. Therefore, the decrease of $P_{app}$ of HePC at 4°C could not be ascribed to the involvement of an energy-dependent transport, but would rather result from a change in membrane fluidity.

Furthermore, to detect the involvement of an energy-dependent mechanism, HePC transport across Caco-2 cell monolayers was studied in conditions inducing energy depletion (Table IB). HePC transport was not affected, since the $P_{app}$ values were 2.69±0.19 and 2.93±0.24×$10^{-6}$ cm/s for control and ATP depletion respectively. This result clearly demonstrated that no active mechanism is involved in the intestinal transepithelial transport of this drug.

## Concentration-dependence

The AP-to-BL transport of HePC across Caco-2 cell monolayers was studied as a function of concentration, from 5 μM to 150 μM of HePC at both 37 and 4°C after a 3-h incubation. Higher concentrations of HePC were not used because they reduced monolayer integrity, as shown by the

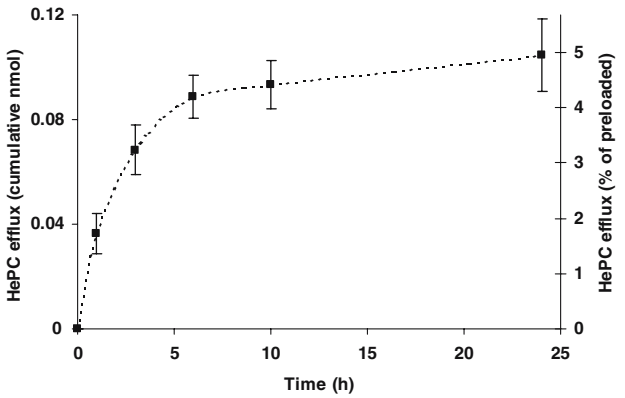


**Fig. 3.** Time course of basolateral HePC efflux from Caco-2 cell monolayers. Cells were kept at 37°C, 5% $CO_2$ and 95% relative humidity during all experiment. Cells were preloaded with [$^3$H]HePC (20 μM, 0.5 μCi per well) added to the apical medium. After a 2-h incubation, cells were washed three times and apical and basolateral compartments were filled with fresh incubation medium at 37°C. The efflux of HePC from basolateral side of Caco-2 monolayers was determinated by taking samples from basolateral compartment at intervals for scintillation counting. Results are the mean±S.D. of three determinations with three different monolayers ($n$=9).

**Table I.** (A) Influence of Temperature on $P_{app}$ of HePC, Mannitol and Testosterone. (B) Influence of ATP Depletion on $P_{app}$ of HePC

| | Compound | $P_{app}$ (±S.D.)×$10^{-6}$ (cm/s)[a] 37°C | 4°C |
|---|---|---|---|
| A | HePC | 2.39±0.47 | 1.34±0.27 |
| | Mannitol | 1.10±0.07 | 0.65±0.01 |
| | Testosterone | 29.02±1.31 | 11.58±0.64 |
| B | HePC—control | 2.69±0.19 | nd[b] |
| | HePC—ATP depletion | 2.93±0.24 | nd[b] |

[a] Results are the mean±S.D. of three determinations with three different monolayers ($n$=9).
[b] not determined

transepithelial electrical resistance and MTT measurements (data not shown). Figure 4 shows that at 37°C the transepithelial transport of HePC was non-saturable over the concentration range studied. Indeed, the amount of HePC crossing the monolayer as a function of concentration gradually increased as the concentration increased. Therefore, the $P_{app}$ increased over the concentration range studied (data not shown). As far as the transepithelial permeation of HePC at low temperature was concerned, Fig. 4 shows that at 4°C, the transport of HePC was significantly reduced compared with that at 37°C, except for concentrations below 20 μM. At 4°C, the relationship between concentration and AP-to-BL permeation of HePC was quasi-linear at the time and doses studied, demonstrating the existence of a non-saturable passive diffusion component for its transport across Caco-2 cell monolayers. In addition, these results showed that the passage of HePC became more sensitive to temperature as the concentration increased, as the difference between 4 and 37°C was much more pronounced at 150 μM than at 20 μM.

### Evaluation of the Transcellular or Paracellular Pathway Involved in HePC Transport

After demonstrating the passive transport of HePC across Caco-2 monolayers, the next step of this study was to determine the extent to which the paracellular and transcellular passive pathways contributed towards the passage of the drug across Caco-2 cell monolayers. Similar approaches have already been used for elucidating the paracellular or transcellular transport mechanisms of various compounds (23–26).

#### *Comparison of HePC with a Paracellular Marker*

To establish whether the transepithelial permeation of HePC was transcellular rather than paracellular, the percentage of added HePC transported into the basolateral compartment was compared with that of mannitol—a hydrophilic molecule which is absorbed exclusively by passive diffusion through the paracellular route (27)—in the same monolayers (Fig. 5). These results demonstrated a linear correlation between HePC and mannitol transport across Caco-2 cell monolayers ($R^2$>0.96). When a low concentration of HePC was added (Fig. 5A), the percentage of mannitol transported was similar to that of HePC, strongly suggesting that this transport was mediated by the paracellular route. Interestingly, in case of incubation with a higher dose of HePC (Fig. 5B), the magnitude of mannitol transported exceeded that of HePC in all cases by about threefold.

#### *Transport After Alteration of the Tight Junction Barrier*

In order to confirm that HePC crosses the intestinal barrier via the paracellular route, its transport across Caco-2 cell monolayers was evaluated after pretreatment with EDTA, a known modulator of paracellular permeability via tight junction opening (22), and compared it with that of mannitol and testosterone. The results are shown in Table II. As expected, after EDTA treatment, the $P_{app}$ of mannitol was considerably increased by approximately 30-fold (from 1.0±0.3 to 30.8±2.2×$10^{-6}$ cm/s) and the TEER value decreased to 31±5%, compared with control, confirming the opening of the tight junctions. Furthermore, Table II shows that EDTA treatment, at the concentration and exposure time studied, did not affect the $P_{app}$ of testosterone, indicating that EDTA treatment had no effect on the passive transcellular pathway in Caco-2 cell monolayers. This observation is in accordance with the literature where it has already been demonstrated that EDTA treatment does not affect the permeability of a transcellularly transported compound (28). As far as HePC was concerned, Table II shows that the $P_{app}$ value increased three-fold in EDTA-treated cell monolayers (7.7±1.2×$10^{-6}$ cm/s) compared with untreated cells (2.5±0.8×$10^{-6}$ cm/s). These data suggest that at least part of the HePC transport across the intestinal epithelium is due to simple diffusion by the paracellular route, through the tight junctions.

## DISCUSSION

Despite the well documented activity of the antileishmanial drug HePC after oral administration, little is known

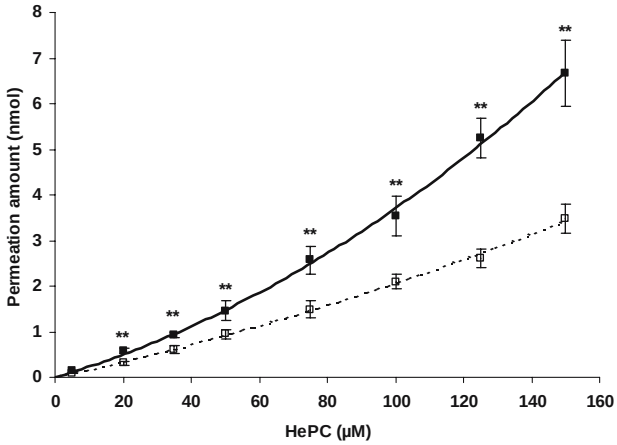


**Fig. 4.** Concentration- and temperature-dependence of [$^3$H]HePC transport across Caco-2 cell monolayers. Cells were incubated with [$^3$H]HePC (0.5 μCi per well) at total concentrations of HePC from 5 to 150 μM. The amount of HePC transported from the apical to the basolateral compartment was measured at 37°C (*filled square*) or 4°C (*empty square*) after a 3-h incubation, and plotted as a function of the amount of HePC added to the apical compartment. Each point represents the mean±S.D. ($n$=9) of four experiments. **$p$<0.01 37 *vs.* 4°C.

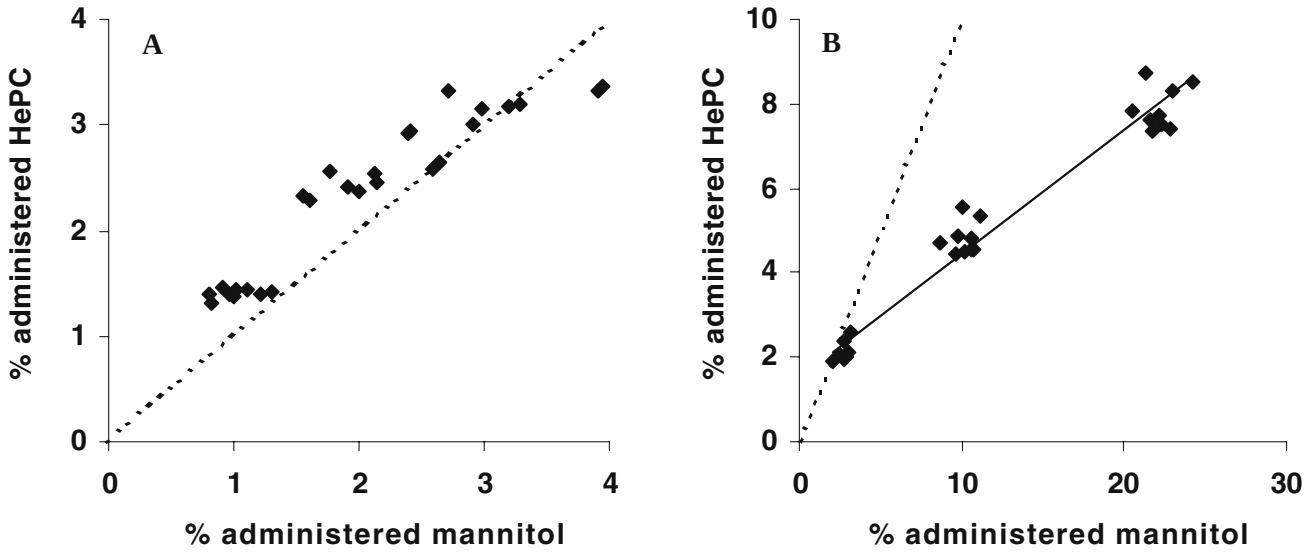


**Fig. 5.** Comparison of transepithelial transport of HePC with that of the paracellular transport marker mannitol in Caco-2 cell monolayers at 37°C. (A) Transport of [$^3$H]HePC (5 μM, 0.5 μCi per well) and [$^{14}$C]mannitol (1.7 μM, 0.05 μCi per well) expressed as the percentage of the administered radiolabeled compound recovered in the basolateral compartment (n=27). (B) Transport of [$^3$H]HePC (100 μM, 0.5 μCi per well) and [$^{14}$C]mannitol (1.7 μM, 0.05 μCi per well) expressed as the percentage of the administered radiolabeled compound recovered in the basolateral compartment ($n$=27). The dashed line represents the percentage of administered HePC that would have been recovered if its transport were equivalent to that of the paracellular marker mannitol.

about the mechanisms of its intestinal absorption. Thus, the purpose of this study was to characterize the mechanism of HePC transport across the human intestinal epithelium. We therefore used the human-derived intestinal epithelial cell line Caco-2 grown on permeable polyester membranes as a validated *in vitro* intestinal transport model system (14,29).

The first objective was to demonstrate whether the HePC transport across the intestinal epithelium involved an active or passive mechanism. The data demonstrated that HePC transepithelial transport in Caco-2 cell monolayers was independent of the transmembrane proton gradient and linear as a function of time. Moreover, HePC transport across Caco-2 cell monolayers in the absorptive (AP-to-BL) and the secretory (BL-to-AP) directions was not significantly different. This bidirectional transepithelial transport suggests that no secretion of HePC by a MDR-mediated efflux mechanism occurs in the Caco-2 cell system. However, it is possible that the absence of active efflux is the result of a false negative. Indeed, we have already reported that HePC can inhibit intestinal *P*-glycoprotein (*P*-gp) activity (20), whereas other authors have demonstrated an interaction between HePC and the *P*-gp (30). It is possible that the inhibition of the *P*-gp by HePC could counterbalance its active efflux by this protein. However, it has to be noted that the use of known *P*-gp inhibitors did not affect the AP-to-BL transport of HePC across the Caco-2 monolayer (data not shown). Taken together, these data strongly suggest that HePC exhibited a non-polarized transport in this *in vitro* intestinal model, and that it would be transported across Caco-2 cell monolayers by simple diffusion–solubility criteria rather than by active transport or facilitated diffusion. To further investigate the absorption mechanisms of HePC, the effect of temperature was determined. HePC transport was studied at low temperature to inhibit a possible energy-dependent mechanism. The transport of HePC across Caco-2 cell monolayers was found to be partially temperature-dependent. Indeed, the $P_{app}$ of HePC was reduced two-fold at 4°C compared with 37°C, as were the $P_{app}$ of mannitol and testosterone, two markers of passive diffusion pathways. Therefore, the reduction of HePC transport at low temperature could not be ascribed to the involvement of an energy-dependent mechanism in the drug transport. Furthermore, it has already been demonstrated that when an active mechanism is involved, the transport of the molecule is almost completely abolished at 4°C compared with 37°C (29,31). In addition, HePC transport was not reduced when energy

**Table II.** Effect of Pretreatment with EDTA on $P_{app}$ of HePC, Mannitol and Testosterone

| | $P_{app}$ (±S.D.)×$10^{-6}$ (cm/s)[a] | | |
|---|---|---|---|
| Compound | Control cells | EDTA-treated cells | Degree of increase |
| HePC | 2.53±0.80 | 7.68±1.19* | 3.0 |
| Mannitol | 1.04±0.25 | 30.83±2.24* | 29.6 |
| Testosterone | 29.02±2.43 | 30.29±2.84 | 1.0 |

[a] All measurements are expressed as mean±S.D ($n$=6).
*$p$<0.01 EDTA-treated *vs.* control

depletion conditions were applied. Taken together, these results clearly demonstrated that HePC transport across Caco-2 cell monolayers occurred by a significant passive permeation rather than by an active mechanism. However, since testosterone is transported by transcellular mechanism, whereas mannitol is transported via the paracellular pathway, these results could not discriminate between these two pathways for HePC transport.

The second objective of this study was to evaluate whether this passive transepithelial permeation of HePC occurred via the paracellular or transcellular route. The data demonstrated an almost linear relationship between the degree of HePC transport and that of the water-soluble paracellular marker mannitol, suggesting that the transport of HePC may be dependent on the permeability of the tight junctions. At a low concentration of HePC, the excellent correlation between the drug and the paracellular transport marker strongly suggests that paracellular route is that taken by HePC. On the other hand, at a higher concentration, HePC strongly enhanced the $P_{app}$ for both HePC and mannitol with increasing HePC concentration. Previous studies have demonstrated that alkylphosphocholines and similar phospholipid-like compounds (i.e. the phosphate ester dodecylphosphocholine, DPC) could enhance *in vitro* paracellular permeability of various epithelial cell lines by opening the tight junctions (32–34). More recently, we have investigated the effects of HePC on the intestinal barrier and shown that HePC also increased *in vitro* paracellular permeability of Caco-2 cells monolayers in a time-, temperature- and concentration-dependent manner (20). Therefore, it is reasonable to assume that HePC could increase its own paracellular passage. As it has been demonstrated that the intrinsic paracellular permeability of drugs decreases with increasing molecular size (24), the observation that the percentage of administered HePC (MW 408) that crossed the Caco-2 monolayers was significantly lower than that of mannitol (MW 182) under these conditions remains consistent with the hypothesis of paracellular transport. Moreover, HePC transport was reduced at low temperature and became more sensitive to temperature change as the concentration increased. This is in accordance with our previous results that the HePC-induced increase in paracellular permeability was less pronounced at 4°C than at 37°C (20). Although the molecular size of mannitol (MW 182) is somewhat lower than that of HePC (MW 408) the relative effect on permeability should be about the same. Therefore, at 4°C, HePC promotes its transport in a less efficient way than at 37°C, since the tight junctions are not opened to such a great extent.

We also showed that an opening of the tight junctions with EDTA induced a concomitant increase in HePC and mannitol transport without affecting the transcellular transport of testosterone. Those results can be compared with studies on the intestinal absorption of sodium dodecyl sulfate (SDS, MW 288), an amphipathic organic anion with a $C_{12}$ aliphatic chain (35). Indeed, SDS was absorbed predominantly by a paracellular pathway, as the absorption rate increased threefold when paracellular junction pore size was increased by the addition of cytochalasin D, another known paracellular penetration enhancer. Taken together, these results clearly provide good circumstantial evidence for the paracellular transport of HePC across this *in vitro* intestinal epithelium model. Since HePC is an amphiphilic molecule which is extremely water-soluble, the intestinal absorption could be mediated by "solvent drag" in the fluid absorbed through the paracellular pathway, as suggested for the intestinal absorption of many hydrophilic drugs and peptides (36–38) and for SDS (35).

Previous studies have demonstrated the good correlation between permeability measured across Caco-2 cell monolayers, solubility and oral bioavailability in humans (24,39,40). These correlation studies were mainly performed with passively transported drugs, and showed that absorption is negligible for drugs with a $P_{app}$ value$<0.1\times10^{-6}$ cm/s and essentially complete for drugs with a $P_{app}$ value$>5\times10^{-6}$ cm/s (41,42). Here, we reported a $P_{app}$ value for HePC of around $2.5\times10^{-6}$ cm/s, consistent with significant transport of HePC across the intestinal epithelium. However, these *in vitro* results are not perfectly in accordance with previous *in vivo* studies on the bioavailability of HePC in rats and dogs which was found to be 82 and 94%, respectively (43). The *in vivo* bioavailability seems higher than the *in vitro* permeability would predict. It should be noted that the role of albumin as a HePC binding protein is not taken into account in the in vitro studies and could contribute to the high bioavailability observed *in vivo*. Moreover, it has been demonstrated that the permeability of the tight junctions in Caco-2 cell monolayers is lower than the average permeability observed in the human intestine *in vivo* (14,15), but that compounds transported by this route *in vivo* are also transported paracellularly in Caco-2 cells. This clearly explains the limited transport in the Caco-2 model compared to *in vivo* absorption, since we have demonstrated that part of HePC crosses the epithelium by the paracellular route.

However, we observed that a large amount of HePC was incorporated into the cell monolayer and was only slowly released. Although these *in vitro* results can be correlated with a previous *in vivo* study on distribution of APCs in mice demonstrating a very remarkable depot effect at the sites of administration, and especially after oral application where more than 80% of the drug still resided in the gastrointestinal tract 5 h after administration (44), this result would appear to be inconsistent with the paracellular pathway for HePC transport. Indeed, a compound which appears to be transported across intestinal mucosa exclusively via the intercellular junctions should not be taken up into the cells. However, HePC, structurally related to naturally occurring phospholipids, is known to be easily incorporated into lipid membranes (12). Moreover, the transporters Lem3p (ligand-effect modulator 3 protein) (45) and LdMT (*L. donovani* putative Miltefosine Transporter) (13,46) have recently found to be required for the inward translocation of HePC into *Saccharomyces cerevisiae* yeast cells and *L. donovani* parasites, respectively. Taking all these observations into account, it is reasonable to suppose that HePC could penetrate the phospholipid bilayer of the apical side of Caco-2 cell, move across the cell in the inner leaflet by lateral diffusion to the basal side of the cell thus reaching the basolateral compartment. Transcellular permeation could therefore occurs in parallel with paracellular permeation. This hypothesis is in accordance with the albumin-dependence of HePC transport demonstrated in this study. Indeed, as reported previously, APCs and ALPs bind to serum proteins

and especially albumin (6,47–49). The strong affinity of HePC to protein components could easily induce an enhancement of HePC transport across Caco-2 monolayer. In case of absence of serum proteins in the basolateral compartment, the avidity of HePC molecules for the membrane surface prevent the HePC efflux from the basolateral side. Therefore, in the Caco-2 cell model, the proportion of HePC molecules that crosses the monolayers through the transcellular pathway was probably reduced compared to the *in vivo* situation. Furthermore, the BL-to-AP permeability was significantly reduced in the presence of albumin in the BL compartment (data not shown), demonstrating the high stability of the HePC-albumin complex, and also suggesting that the part of HePC transported by the paracellular route is driven by a concentration gradient where only free molecules of HePC could be transported across the monolayer.

## CONCLUSION

In conclusion, the use of the *in vitro* model consisting of Caco-2 cells grown on permeable support emphasized the paracellular contribution to HePC transport. Although this contribution may not be reponsible for all transport of HePC across the intestinal epithelium *in vivo*, this study demonstrates for the first time that (1) HePC transport across the intestinal barrier involves a passive mechanism rather than a carrier-mediated one and (2) that HePC promotes its own transport throught the intercellular spaces via an opening of the tight junctions (20). This study throws new light on the passage of HePC across biological barriers and helps to explain its remarkable oral absorption in humans.

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