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Modulation of intestinal barrier properties by miltefosine

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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)

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[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.