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Low *in vitro* permeability of the cyanotoxin microcystin-LR across a Caco-2 monolayer: With identification of the limiting factors using modelling

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ABSTRACT

Microcystins (MCs) are toxins produced by several cyanobacteria species found worldwide. MC-LR is the most frequent. Here, we used the human Caco-2 cell line grown on semi-permeable filter supports as an *in vitro* model for determining MC-LR intestinal bidirectional transport. In this study, there was very low and time-dependent apparent permeability of MC-LR. To identify the limiting factors involved in the low permeability of MC-LR, a mathematical model was constructed to get physiologically relevant and informative parameters. The apical-to-basolateral transport was characterised by a rapid and substantial decrease in apical MC-LR concentrations (24–40% of the initial amount). In the basolateral compartment, the concentrations increased slowly after a lag time, but represented only a small fraction of the loaded concentrations (0.3–1.3%) after 24 h. This weak permeability was mainly due to a low clearance of efflux (from the cellular to the basolateral compartment) and effective secretion (from the cellular to the apical compartment). During the basolateral-to-apical transport, we observed a slow decrease in basolateral concentrations and a rapid increase in apical concentrations. In conclusion, modelling has the potential to highlight the key mechanisms involved in the complex kinetics of toxin transport.

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1. Introduction

Microcystins (MCs) are freshwater toxins produced by several cyanobacteria species found worldwide that can cause intoxications mainly through consumption of water. The ingestion of MCs is of concern for human but also animal health (Sivonen and Jones, 1999). MCs are heptapeptides comprising approximately 90 structural congeners (Welker and Von Döhren, 2006) with sizes ranging from 900 to 1100 Da (Sivonen and Jones, 1999). They have a common cyclic structure usually containing three D-amino acids, two variable L-amino acids, two unusual residues, N-methyldehydroalanine, and a unique C_{20} amino acid residue, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,E-dienoic acid (Adda) (Carmichael et al., 1988; Rinehart

Abbreviations: A, apical; ADME, absorption distribution metabolism excretion; B, basolateral; C_a, apical concentration; C_b, basolateral concentration; C_c, cellular concentration; CL, clearance; CL_{abs}, clearance of absorption; CL_{eff}, clearance of efflux; CL_{inf}, clearance of influx; CL_{sec}, clearance of secretion; IVIVE, *in vitro-in vivo* extrapolation; K_{activ}, constant of activation; K_{met}, biotransformation constant; logP, logarithm of the ratio of the concentrations of the un-ionized solute in the solvents; LOQ, limit of quantification; MC, microcystin; MC-LR, microcystin-LR; Nb_{cell}, number of cells; OATP, organic anion transporting polypeptide; Pgp, permeability glycoprotein; PP, protein phosphatase; PPc, catalytic subunit of PP; Q_a, apical amount; Q_b, basolateral amount; Q_c, cellular amount; TEER, transepithelial electrical resistance; V_a, apical volume; V_b, basolateral volume; V_c, cellular volume; V_{cell}, volume of a cell.

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et al., 1994; Harada, 1996). MCs contain some hydrophilic functional groups, such as a carboxyl group, and frequently the guanidine group of an arginine residue, and some hydrophobic components, such as the Adda residue. Microcystin-LR (MC-LR), containing leucine and arginine as the two variable L-amino acids, is the most frequent MC.

MCs are potent inhibitors of serine/threonine protein phosphatases (PPs), especially PP1 and PP2A (Honkanen et al., 1990; Runnegar et al., 1995; Toivola et al., 1997; MacKintosh, 1999; Hastie et al., 2005). Inhibition of PPs results in the hyperphosphorylation of the threonine and serine residues of numerous proteins, which in turn deregulates crucial cellular processes (Eriksson et al., 1990a; Yoshizawa et al., 1990; Ohta et al., 1992; Wickstrom et al., 1995; Batista et al., 2003). The toxicity of MC-LR in mice is 100 times lower after oral treatment (median lethal dose LD_{50}, 5 mg/kg) (Fawell et al., 1994) than after intraperitoneal injection (LD_{50}, 50 μg/kg) (Kuiper-Goodman et al., 1999). The difference in toxicity between these two routes of exposure cannot be assessed without complementary data on MC-LR toxicokinetics.

Due to its structure and amino-acid composition, MC-LR is hydrophilic (log P, 2.16), ionised at pH 3.3 and with a molecular weight of 997 g/mol explaining the apparent inability of MC-LR to cross lipid bilayers via passive diffusion (Vesterkvist and Meriluoto, 2003). In rodent hepatocytes and chicken enterocytes, absorption involving organic anion transporting polypeptides (OATPs) has been suggested (Runnegar et al., 1991; Falconer et al., 1992). Moreover, in rat hepatocytes, absorption of microcystins is decreased by bile acids (cholate, taurocholate) and other OATP substrates (cyclosporine A, rifampicin) (Eriksson et al., 1990b; Runnegar et al., 1991, 1995). In addition to absorption transporters, secretion proteins may be involved in MC-LR transport, such as the permeability glycoprotein (Pgp) (de Souza Votto et al., 2007; Contardo-Jara et al., 2008; Amé et al., 2009). Therefore, for MC-LR, crossing the intestinal epithelium likely involves transporter systems rather than passive paracellular absorption. Additional information is needed on MC-LR intestinal toxicokinetics, i.e. absorption, distribution, metabolism and excretion (ADME).

To explore the transport systems involved in the transepithelial permeability of MC-LR, the human colon adenocarcinoma cell line Caco-2 grown on semi-permeable filter supports (van Breemen and Li, 2005; Hubatsch et al., 2007) was used as an *in vitro* model. Differentiated Caco-2 cells exhibit functional and morphological characteristics similar to enterocytes. Given that MC-LR kinetics are complex, a mathematical model, describing the concentration-time profiles, was constructed to sort out the key mechanisms involved.

2. Materials and methods

2.1. Chemicals

MC-LR (purity certified higher than 95% by an HPLC method) was purchased from Novakits (Nantes, France). Ethanol was purchased from Carlo Erba (Val de Reuil, France). Cell culture products and Hank's balanced salt solution (HBSS) were purchased from Gibco (Invitrogen™, Cergy-Pontoise, France).

2.2. Cell culture

Caco-2 cells (American Type Culture Collection HTB37™) were purchased from LGC Standards (Molsheim, France). The cells were maintained in minimum essential medium (MEM) containing 1 g/L glucose, Earle's salts and L-glutamine (MEM GlutaMAX™) with 10% foetal bovine serum, 1% non-essential amino acids, 50 IU/mL penicillin and 50 μg/mL streptomycin in 5% CO_2 at 37 °C. For the transport studies, cells (passage 32–34) were seeded in 12 well plates on polyester membrane inserts (0.4 μm pore diameter, 12 mm insert, Corning, USA) at 6×10^4 cells/cm^2. Culture medium was changed three times a week. Cells were used for transport experiments at day 25–27 post-seeding (Sambuy et al., 2005; Huguet et al., 2013).

2.3. Bidirectional transport experiments

After rinsing with transport buffer (HBSS, HEPES 5 mM, pH 7.4), the Caco-2 cell monolayers were incubated for 30 min at 37 °C with 300 and 1000 μL transport buffer in the apical (A) and basolateral (B) compartment, respectively. The transport buffer from the donor compartment (A compartment during A-to-B transport and B compartment during B-to-A transport) was replaced with MC-LR solutions of 1, 10, 48 or 75 μM in transport buffer (prepared in glass vials with respectively 300 μL and 1000 μL in apical and basolateral compartment). A preliminary experiment to check binding to plastic (MC-LR solutions incubated for 1 h in transwell plates without cells) showed that loss was negligible (data not shown). During transport experiments, the plates were incubated at 37 °C and shaken at 100 rpm on a titre plate shaker 4625 (Thermo Scientific, Dubuque, Iowa, USA). At different time points (0.5, 1, 2, 4, 6 and 24 h for A-to-B transport experiments and 0.5, 1, 2, 4, and 6 h for B-to-A transport experiments), the total volume of each compartment was harvested into glass vials and stored at −20 °C until analysis. Therefore, the same well was not used for different sampling times (each well was used for a single measurement) and four independent biological replicates (different passages) were available per sampling time.

The final volume of ethanol added to the donor compartment was set to 2.7% for all MC-LR concentrations. Both negative (100% HBSS) and solvent (2.7% ethanol) controls were also included on each plate to check the influence of HBSS and ethanol (without MC-LR) on the integrity of the monolayer. The integrity of the Caco-2 monolayers was controlled by trans-epithelial electrical resistance (TEER) measurements, before and after MC-LR treatment. Only the transwells with a TEER >250 Ohm/cm^2 before and after MC-LR treatment were used and sampled.

2.4. Analytical methods

An analytical method was developed for this study. Briefly, chromatographic separations were performed on an Accela liquid chromatography U-HPLC system

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