Intestinal transport of Cylindrospermopsin using the Caco-2 cell line

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Abstract

Cylindrospermopsin (CYN) is a cyanotoxin produced by various cyanobacterial species. It is a water soluble zwitterion, stable at extreme temperatures and pH. Despite the main route of exposure to CYN is through drinking water and food, there is a lack of data concerning its intestinal absorption and the mechanisms implicated.

The aim of this study was to characterize the mechanisms involved in the intestinal absorption of CYN, using Caco-2 human cell line as a model of the intestinal epithelium. The results obtained in the present work increases the limited knowledge regarding CYN transport across the intestinal epithelium and identifies the paracellular route as an important pathway in CYN absorption. A minor carrier-mediated transcellular transport has been evidenced. This transport is not affected by low temperatures, suggesting that an active mechanism is not involved. Moreover, the transport through the intestinal monolayer is H^+ and GSH dependent and Na+ independent. The transport characteristics elucidated in this study prepare the ground for future studies directed at identifying transporters involved in the intestinal absorption of this toxin.

Keywords: Cylindrospermopsin; Caco-2; intestinal transport; glutathione, biliar salts.

1. Introduction

Cylindrospermopsin (CYN) is a cyanotoxin produced by a number of cyanobacterial species including *Cylindrospermopsis raciborskii* (Ohtani et al., 1992), *Umezakia natans* (Terao et al., 1994), *Aphanizomenon ovalisporum* (Shaw et al., 1999), *Raphidiopsis curvata* (Li et al., 2001), *Anabaena bergii* (Stüken et al., 2006), *Aphanizomenon flos-aquae* (Preussel et al., 2006), and *Anabaena lapponica* (Bazin et al., 2010). The toxin structure consists of a tricyclic guanidine linked to hydroxymethyluracil group (Ohtani et al., 1992). CYN is a zwitterion highly water soluble (Sivonen and Jones, 1999), being stable to extreme temperatures and pH (Chiswell et al., 1999). Exposure to CYN is likely to happen since the 90% of this cyanotoxin is released from cyanobacterial cells to the water (Chiswell et al., 1999; Rücker et al., 2007).

Human can be intoxicated by this cyanotoxin mainly by the oral route through the ingestion of contaminated drinking water (Kuiper-Goodman et al., 1999; Gutiérrez-Praena et al., 2012) and also by food contaminated with CYN (Kittler et al., 2012; Gutiérrez-Praena et al., 2013). Two severe human poisoning events have been reported. In North Queensland (Australia), a bloom of *Cylindrospermopsis raciborskii* in a drinking water reservoir resulted in human poisoning undergoing damage in liver and kidney (Hawkins et al., 1985). Also, in the Brazilian dialysis clinic tragedy in 1996, the presence of CYN and microcystins in dialysis water resulted in more than 50 deaths (Carmichael et al., 2001).

Despite the oral route is the main pathway of human exposure to CYN, there is a lack of data concerning the intestinal absorption of this cyanotoxin. Regarding the mechanisms of transport implicated in the absorption, due to the small size of the molecule, a limited passive diffusion has been suggested (Runnegar et al., 2002). In addition, some studies have indicated the participation of a facilitated transport mechanism (Froscio et al., 2009) and an active transport, the latter making use of bile acid transport systems (Chong et al. 2002). In order to better understand the mechanisms of action of this toxin and to

attempt to reduce these toxic effects, it is necessary to characterize its intestinal absorption.

The study of the intestinal absorption can be addressed using *in vivo* or *in vitro* models. However, due to the ease and accuracy of *in vitro* models, they are extensively used, being the most employed the Caco-2 cell line (Rocha et al., 2013). These cells are derived from a colon adenocarcinoma which differentiate spontaneously after 14-15 days of culture (Calatayud et al., 2011). When Caco-2 cells are differentiated they exhibit morphological and functional features of mature enterocytes (Hidalgo et al., 1989), and they can even express some transporter also present in the human small intestine (Maubon et al., 2007). All these features have converted Caco-2 cells into a suitable model for studying the mechanisms of intestinal absorption. Indeed, this model is currently used for *in vitro* study of absorption of pharmaceuticals (Sevin et al., 2013; Nohr et al., 2014) and minerals (Ca, Fe, Zn) (Calatayud et al., 2011); however, it is not extensively used in the study of absorption and bioavailability of cyanotoxins. The use of Caco-2 monolayers has been practically restricted to microcystins (Zeller et al., 2011; Henri et al., 2014), and there is only an approach to the evaluation of the intestinal permeability of CYN (Fernandez et al., 2014).

Considering the lack of data in this field, the present work aimed to investigate the mechanisms involved in the intestinal absorption of CYN using Caco-2 human cells as a model of intestinal epithelium. In order to address this objective, transport assays were carried out both in absorptive and secretory directions. It has been evaluated the participation of paracellular and transcellular (active or passive) pathways, analysing the influence of parameters such as concentration of H^+, Na^+ and GSH. Taking into account the information obtained in these assays and the data from literature, the participation of some transporters has also been investigated by means of assays of competitive inhibition using natural ligands of these transporters (taurocholate and L-carnitine).

2. Materials and methods

2.1. Cyanotoxin standard.

The cyanotoxin cylindrospermopsin standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Stock solution of CYN (100 µg/mL) was prepared in sterilized milliQ water and maintained at 4ºC until analysis.

2.2. Culture of Caco-2 cells.

Caco-2 cell line, derived from a human colon carcinoma, was acquired from the American Type Culture Collection (ATCC® HTB-37, Manassas, VA, USA). Cells were cultured in 75 cm^2 flasks using Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) at pH 7.2, supplemented with 10% (v/v) of heat-inactivated fetal bovine serum, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 100 U/ml of penicillin, 0.1 mg/ml of streptomycin and 0.0025 mg/l of amphotericin B (DMEMc); and incubated at 37º C and 5% flow of CO_2. The medium was changed every 2–3 days. When the cell monolayer reached 80% confluence, they were detached with a solution of trypsin (0.5 mg/mL) and EDTA (ethylene diamine tetraacetic acid, 0.2 mg/mL) and reseeded at a density of 5×10^4 cells/cm^2. The cells were used between passages 21 and 34. All the reagents used were acquired from Hyclone (Fisher, Spain).

For CYN transport assays, Caco-2 cells were seeded on 6-well plates with inserts with a polyester membrane (diameter 24 mm, pore size 0.4 µm; Transwell®, Corning, Cultex, Spain). The porous support of the insert in which Caco-2 cells were seeded separates the well into two compartments: apical (upper) and basolateral (lower). The apical (A) compartment represents the intestinal lumen and the basolateral (B) compartment the serous compartment (blood or lymphatic vessels). The cells were seeded at a density of 7.5×10^4 cells/cm^2 and they were supplemented with 1.5 mL of DMEMc in the apical chamber and 2 ml of DMEMc in the basolateral chamber. The medium was changed

every 2–3 days until cell differentiation was achieved (14-15 days post seeding). The formation of Caco-2 monolayers was evaluated during the differentiation by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS voltohmmeter (Millipore Corp, Madrid, Spain). The cells were considered suitable for the assays when the TEER values were greater than 250 Ω cm^2.

2.3. Cell viability assays.

The effect of various concentrations of CYN on the viability of Caco-2 cells was evaluated by using sodium resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma). The cells were seeded at a density of 2.5×10^4 cells/cm^2 in 24-well plates and supplemented with 1 mL of DMEMc. After differentiation took place, cells were exposed to various concentrations of CYN (0.8, 2, 5, 10, 20 µg/mL prepared in DMEMc without fetal bovine serum) for 24 and 48 h. After exposure, the medium was withdrawn and the culture was washed with phosphate buffered saline (PBS, Hyclone). Then 500 µL of resazurin solution (10 µg/mL in DMEMc without serum) were added and it was incubated for 2 h at 37°C, 5% CO_2 and 95% relative humidity. A volume of 100 µL for each condition studied was transferred to a 96-well plate and resazurin reduction was measured colorimetrically (570 and 600 nm) using a PowerWave HT microplate scanning spectrophotometer (BioTek Instruments, USA).

2.4. Transport assay: Calculation of permeability coefficients (P_{app}).

The transport assay was performed in Hanks' balanced salt solution with $NaCO_3$ (HBSS) (Hyclone) supplemented with 10 mM of HEPES (pH 7.2). The study was conducted in apical-basolateral direction (A-B), absorptive direction, and in the basolateral-apical direction (B-A), the secretory one. The standard solutions of CYN (0.8 µg/mL) prepared in HBSS-10 mM HEPES were added to the donor compartment (apical or basolateral, depending on the transport direction). At the established times (15, 30, 45, 60, and 120

min), the contents of the acceptor compartment were totally removed and replaced by an equal volume of fresh medium. CYN was determined in aliquots of the acceptor medium removed at each time and in the donor medium removed at the end of the experiment following the protocol described in section 2.12.

The apparent permeability coefficients (P_{app}) were calculated by using equation 1.

$$P_{app} = (dC/dt)\ (V_r/AC_o) \quad \text{(Eq.1)}$$

where:

dC/dt is the flow (µg/s) determined by the linear slope of the equation that governs the variation in the concentrations of CYN, corrected with dilution, against time.

V_r is the volume of the acceptor compartment (2 mL in A-B direction or 1.5 mL in B-A direction).

A is the surface occupied by the cell monolayer (4.67 cm^2).

C_o is the initial concentration of CYN in the donor compartment (µg/mL).

The efflux ratio (Er) was calculated using equation 2,

$$E_r = P_{app}(B\text{-}A)/P_{app}(A\text{-}B) \quad \text{(Eq. 2)}$$

where:

$P_{app}(B\text{-}A)$ is the apparent permeability coefficient in the basolateral-apical direction (cm/s).

$P_{app}(A\text{-}B)$ is the apparent permeability coefficient in the apical-basolateral direction (cm/s).

2.5. Study of paracellular transport of CYN.

The participation of the paracellular pathway in transport of CYN was evaluated by modulating the cell junctions in both directions (A-B and B-A). For this purpose, the cell monolayer was incubated with 5 mM EDTA in PBS free of Ca^{2+} and Mg^{2+} (Hyclone) for 5 min. Then, the standard of CYN (0.8 mg/L), prepared in medium consisting of 50% of

HBSS free of Ca^{2+} and Mg^{2+} (Hyclone) and 50% of HBSS with Ca^{2+} and Mg^{2+}, both supplemented with 10 mM HEPES, was added to the apical compartment. The acceptor medium was collected at various times (15, 30, 45, 60 and 120 min) and the concentration of CYN was determined in order to evaluate the P_{app} in both directions (equation 1). At the same time, the efficiency of the EDTA in modulating the cell junctions was monitored by determining the P_{app} of Lucifer Yellow (LY) before and after the EDTA treatment. LY is a fluorescent compound transported mainly across intercellular junctions which evidence the integrity of the monolayer.

2.6. Effect of temperature on transport of CYN.

The study of CYN transport at 4°C and 37°C was conducted in the A-B and B-A directions. For this study, 0.8 µg/mL of CYN prepared in HBSS supplemented with 10 mM HEPES was added to the donor compartment. At various times (30, 45, 60, 90 and 120 min), the medium was removed from the acceptor compartment and replaced with the same quantity of fresh medium. The concentration of CYN was quantified in the aliquots that were removed and the P_{app} (equation 1) was determined.

2.7. Effect of pH on permeability of CYN.

The effect of pH on transport of CYN (0.8 µg/mL) in the A-B direction was studied with a pH in the acceptor/donor compartments of 5.5/7.2 and then compared with the assays previously made at pH 7.2/7.2. The medium at pH 5.5 was prepared with HBSS supplemented with 20 mM o-2-(N-morpholine) ethanesulfonic acid (MES, Sigma), whereas the medium at pH 7.2 was prepared with HBSS supplemented with 10 mM HEPES. At various times (30, 45, 60, 90 and 120 min) the acceptor medium was collected and, after determining the concentration of CYN, the P_{app} was calculated (equation 1).

2.8. Influence of Na^+ ions and GSH upon transport of CYN.

The influence of Na^+ ions was analyzed in A-B direction. A control medium was prepared [10 mM HEPES, 130 mM NaCl (Panreac), 10 mM KCl (Panreac), 1 mM $MgSO_4$ (Sigma), 5 mM glucose (Panreac) and 1 mM $CaCl_2$ (Panreac)], together with a medium without NaCl in which the latter salt was replaced by an equimolar concentration of choline chloride [$(CH_3)_3N(Cl)CH_2CH_2OH$, Sigma]. The CYN treatment (0.8 mg/L) was prepared in control medium or in medium without NaCl, and was added to the apical compartment (1.5 mL). Control medium (2 mL) was added to the basolateral compartment.

The influence of GSH upon the transport of CYN in the A-B direction was evaluated by co-exposing Caco-2 cells to 0.8 mg/L CYN and GSH (Sigma) at various concentrations (0.1, 1, 10 mM) prepared in HBSS with 10 mM HEPES.

After 120 min, apical and basolateral media were collected and the concentration of CYN was determined (section 2.12).

2.9. Inhibition studies of CYN transport with taurocholate and L-carnitine.

The inhibition assays were carried out at pH 7.2 for L-carnitine and at pH 5.5 on the apical side for taurocholate. The cell monolayer was preincubated with the corresponding inhibitor added to the apical compartment: 0.2mM sodium taurocholate or 0.012 mM L-carnitine (Sigma, Spain). Then, without eliminating the inhibitor, we added the standard solutions of CYN (0.8 mg/L final concentration). At various times (30, 60 and 90 min), the medium was removed from the acceptor compartment and replaced with the same quantity of fresh medium. The concentration of CYN was quantified in the aliquots that were removed and the P_{app} (equation 1) was determined.

2.10. Monolayer integrity during the transport assays.

Monolayer integrity was verified by measuring TEER at different times and determining the apparent permeability coefficient (P_{app}) of LY. This compound was added at a

concentration of 100 µM to the apical side and the transport to the basolateral side was measured at different timepoints during the experiment using a microplate reader (PolarSTAR OPTIMA reader, BMG-Labtech, Germany) at an excitation/emission wavelength of 485/520 nm. The transport assays were only considered valid if, a) the P_{app} values for LY at the end of the assay were not superior to 2×10^{-7} cm/s, and b) the TEER values did not vary by more than 25% from those observed before the beginning of the experiment.

2.11. Evaluation of the participation of passive transcellular transport.

The experiments were performed using parallel artificial membrane permeability assays (PAMPA) with Multiscreen® filter 96 well-plates (Millipore, Spain). A 1% (w/v) solution of lecithin (Sigma) in dodecane (Merck, Spain) was prepared, sonicated to ensure complete dissolution, and carefully added to each donor plate well (5 µL). Immediately after application of the lecithin/dodecane solution, 150 µL of CYN (10 mg/L) prepared in 5% (v/v) dimethylsulfoxide (DMSO, Sigma) in PBS at pH 7.2 was added to the wells of the donor plate. As control verapamil hydrochloride (Sigma) at a concentration of 200 µM was used. Receiver plates with 300 µL of 5% DMSO in PBS were then coupled with the donor plates, and the resulting plate assemblies were incubated at room temperature without agitation for 16 hours in a sealed container with wet paper towels to avoid evaporation.

After incubation, samples from the donor and receptor plates were recovered for analysis of CYN contents. The concentrations of verapamil in the acceptor and donor compartments were determined using a UV-VIS spectrophotometer (Infinite M200, Tecan, Austria) at a wavelength of 279 nm. The permeability coefficient across the artificial membrane (P_{am}) was calculated using the equation described by Sugano et al. (2001):

$$P_{am} = -2.303 \times (V_{dn} \times V_{ac} / V_{dn} + V_{ac}) \times (1/S \times t) \times \log (1 - \textit{flux}\ \%/100)$$

$$flux\ \% = (C_{ac}/C_{ref}) \times 100$$

where V_{dn} is the volume of the donor compartment (0.15 mL), V_{ac} is the volume of the acceptor comportment (0.3 mL), C_{ac} is the quantity of CYN in the acceptor well at the end of the experiment (ng), C_{ref} is the quantity of CYN added to the donor well (ng), S is the membrane area (0.3 cm^2), and t is the incubation time (57.600 s).

2.12. Determination of CYN

The cyanotoxin content was determined by the method described by Guzmán-Guillén et al. (2012) with slight modifications avoiding the clean-up procedure. Briefly, chromatographic separation was performed using a Perkin-Elmer Series 200 HPLC system coupled to an Applied Biosystems QTRAP LC-MS/MS system (Foster City, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQLIT) mass spectrometer equipped with an electrospray ion source. The analytical column was a Zorbax Sb-Aq column (150x2.1 mm) with a particle size of 3.5 µm (Agilent Technologies). The flow rate was 0.2 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of water and methanol with 0.05% trifluoroacetic acid (v/v). The injection volume was 20 µl, and the elution profile was 0% methanol (1 min), linear gradient to 90% methanol (10 min), 90% methanol (5 min), and finally 0% methanol (5 min).

2.13. Statistical analysis.

All the treatments were performed at least in triplicate. The statistical analysis was done using one-way ANOVA with the Tukey HSD post hoc multiple comparison test or using the Student t-test (SigmaPlot version 12.0). Statistical significance was accepted for $p<0.05$.

3. Results

In order to set the concentrations to be used in the transport study, a preliminary cell viability assay was carried out (section 2.3). Caco-2 cells exposed to up to 20 µg/mL of CYN presented viabilities above 95% with respect to non-treated cells (data not shown). For the transport assays the concentration selected was 0.8 µg/mL, which was the lowest concentration that could be detected analytically without uncertainty. Furthermore, no remarkable differences were observed in the TEER and LY permeability values in all the assays carried out, therefore the integrity of the monolayer was assured (section 2.10).

3.1. Transport and permeability coefficients in the A-B and B-A directions.

The results obtained after exposure of Caco-2 differentiated monolayers to CYN over 90 minutes indicate that CYN is transported in the absorptive direction (A-B) in a lesser extent than in the secretory direction (B-A) (Figure 1). Transport of CYN in both cases are very limited, only a 1.5% of CYN is transported in A-B direction and 2.6% in B-A direction, and a saturable component is not observed.

The low transport rate is confirmed with the P_{app} values obtained in both directions. The apparent permeability coefficient is a parameter that indicates the rate of transport across a cell monolayer. It is very a useful for making comparisons among results obtained in various transport conditions, since the values are corrected by the surface of the monolayer, the exposure time and the concentration of the compound (Rocha et al., 2013). P_{app} in A-B direction is 3.45×10^{-7} cm/s, whereas the coefficient in the secretory direction is slightly greater, 6.41×10^{-7} cm/s. The efflux ratio is greater than the unity, 1.86 ± 0.14. This corroborates that elimination of CYN towards the lumen (B-A direction) is favoured with respect to absorption (A-B direction), as the transport curves (Figure 1) and the P_{app} indicate. According to Hubatsch et al. (2007), efflux ratios higher than 1.5 suggest the participation of active secretory mechanisms.

3.2. Study of participation of the paracellular pathway in transport of CYN.

The effect of altering the intercellular junctions upon the permeability of a compound is used to determine participation of the paracellular route (Gan et al., 1993). If the permeability of a given solute increases significantly upon opening the intercellular junctions, then the paracellular route is considered relevant in its uptake. In the present study, EDTA, a calcium chelator, was added in order to modify the cell junctions. Figure 2 shows P_{app} values of CYN in the A-B and B-A directions when Caco-2 cells are treated with EDTA and before this treatment. The treatment with EDTA produces considerable opening of cell junctions, which has been confirmed by the raise in P_{app} of LY (increase of 32 ± 5%) and the reduction in TEER values (decrease of 83 ± 4%). Opening of intercellular junctions significantly increases CYN permeability in both directions, about 10 times in absorptive direction and around 0.7 times in secretory direction. These results clearly indicate that the paracellular pathway is involved in transport of CYN, especially in the process of absorption from the lumen (A-B direction).

3.3. Influence of temperature on permeability of CYN.

Energy-dependent cell processes are minimized at 0-4ºC, and therefore a decrease in transport on lowering the temperature may be indicative of the participation of active transport. No significant changes are observed in the permeability coefficient in A-B direction obtained at 4°C (3.45 ± 0.6 $\times 10^{-7}$ cm/s) in comparison to the values obtained at 37°C (4.20 ± 0.05 $\times 10^{-7}$ cm/s) (Figure 3). This finding suggests that no energy-dependent transport is involved in the process of absorption of CYN or that it is not relevant. However, in the B-A direction a significant reduction in transport at 4ºC is observed (P_{app}= 5.02 ± 0.5 $\times 10^{-7}$ cm/s) when compared to the permeability coefficient achieved at 37ºC (P_{app}=6.41 ± 0.2 $\times 10^{-7}$ cm/s) (Figure 3). A possible participation of energy-dependent carrier in the secretory direction is expected, which confirms the result of the efflux ratio coefficients (section 3.1).

3.4. Effect of pH on permeability of CYN.

Throughout the intestinal tract pH varies depending on the section of the intestine (Nugent et al., 2001). The influence of an acidic pH in the apical compartment in the transport of CYN in A-B direction is shown in Figure 4. The transport of CYN significantly increases at pH 5.5 in comparison to a neutral pH in both compartments (7.2/7.2). The influence of pH on the transport in the gastrointestinal tract may be due to various causes. First, the absorption by passive diffusion depends on the fraction of a compound presents in non-dissociated form (Kristl, 2009). If the variations in pH affect the ionization state, the transport could be modified. This is not the case of CYN, that it is a zwitterion in the range of pHs of the small intestine (5.5-7). Moreover, there are transporters that depend on the proton concentration; this could be the reason for the increased transport of CYN at acidic pHs, the participation of a H^+ dependent carrier.

3.5. Influence of GSH and Na^+ ions upon transport of CYN.

The presence of GSH and Na^+ has been pointed out as a driving force for some transporters which has been pointed out in the scientific literature that may participate in CYN uptake.The influence of GSH in the transport of CYN is represented in Figure 5. The results show that the presence of GSH in the apical medium significantly enhances the transport of CYN in comparison to the control without GSH. A concentration-dependent increase in the transport of CYN when Caco-2 cells are co-exposed with GSH is observed.

Regarding the influence of Na^+ ions, the absence of Na^+ does not decrease the transport of CYN in A-B direction, indicating the absence of a Na^+ dependent transport mechanism (data not show).

3.6. Influence of taurocholate and L-carnitine on the permeability of CYN.

These two compounds have been evaluated in order to determine whether their presence reduces the A-B passage of CYN, by competition for the same transport mechanisms. Taurocholate is transported by bile salts carriers, previously suggested as possible carriers of CYN (Chong et al., 2002). Meanwhile, L-carnitine is a substrate of the organic cation/zwitterion transporters OCTN (Koepsell, 2013), which transport zwitterions as CYN.

Pre-incubation with these two compounds and posterior co-exposure with CYN does not lead to modifications on the permeability coefficients of the cyanotoxin with respect to the cells not treated with these inhibitors (data not shown). These results suggest that transport of CYN through this type of transporters does not occur or it is marginal.

3.7. Evaluation of transcellular passive diffusion of CYN (PAMPA).

This assay uses an artificial membrane made of lecithin and dodecane, which allows identifying the participation of a transcellular passive transport since carrier-mediated and paracellular transport are absent (Sugano et al, 2010). Hence, PAMPA shows trends in the ability of a compound to permeate membranes through passive diffusion (Kansy et al., 1998). The flow values for verapamil, a lipophilic compound that crosses cell membranes mainly through passive diffusion, are high (64.5 ± 2.9%); however, CYN flow values are negligible (data not shown), as might be expected given its hydrophilic character. Therefore, we can conclude that this cyanotoxin does not cross the membrane by simple diffusion through the lipid bilayer or the contribution of this transport mechanism is irrelevant.

4. Discussion

Intestinal absorption plays an important role in the toxicity of contaminants exhibiting the oral pathway as the major route of entry into the body, as instance CYN. Therefore, studies to elucidate the mechanisms involved in the absorption process are necessary

for a better understanding of risks that these compounds can exert after intake. In order to explore the intestinal uptake of compounds, *in vitro* studies provide an interesting alternative to *in vivo* studies (Calatayud et al., 2011). There are several studies that correlate data of apparent permeability coefficients obtained *in vitro* using Caco-2 cell model with the human intestinal absorption rate (Artursson and Karlsson, 1991; Yee, 1997). Hence, the evaluation of this parameter can provide us preliminary information of what might be occurring *in vivo*, although in many cases, extrapolation is difficult (Tavelin et al., 2003).

According to Yee (1997), $P_{app} < 1\times10^{-6}$ cm/s in Caco-2 cells indicates a low *in vivo* absorption (0-20%), P_{app} between $1\text{-}10\times10^{-6}$ cm/s suggests a moderate absorption (20-70%), and $P_{app} > 10\times10^{-6}$ cm/s reveals a high *in vivo* absorption (70-100%). Comparing the results obtained in the present work for CYN in the A-B direction using Caco-2 cells, the absorption *in vivo* would be generally low (<20%). This fact corroborates the results obtained previously by Fernández et al. (2014) using the same cellular model, reporting a limited passage of the toxin through the intact intestinal epithelium. Since no toxicokinetic data performed on laboratory animals is available, the results obtained in the present work cannot be compared with any *in vivo* data.

Solute transport through the intestinal epithelium can occur by two pathways: transcellular or paracellular through intercellular junctions. Paracellular transport involves only passive diffusion mechanism, whereas transcellular transport can be mediated by passive, facilitated or active processes (Sugano et al., 2010). The studies performed in the present study show a significant increase in the permeability of CYN with the opening of intercellular junctions, especially in absorptive direction, indicating that this toxin is transported by paracellular pathway. The increases in the transport of CYN observed after opening the paracellular passage are similar or higher to those observed for LY, compound mainly transported through this route. This finding suggests that this pathway is important in the transport of CYN across the intestinal epithelium.

This would also explain why the values of P_{app} of CYN in absorptive direction deduced using Caco-2 cells are low. The monolayer formed by Caco-2 cells has tighter cell junctions than those in the human small intestine (Tavelin et al., 2003), and therefore it may not be the best model for studying the bioavailability of substances that move paracellularly, since underestimation of the intestinal transport could be observed.

Besides the paracellular transport, a transcellular pathway could also participate in CYN transport. The evaluation of the possible participation of transcellular transport by passive diffusion using artificial lipid membranes shows that this type of transport does not occur or is negligible (section 3.7). This was an expected finding considering the hydrophilic nature of this cyanotoxin. In the case of the transcellular transport mediated by carrier (active or facilitated), initially the assays were focused on the assessment of the participation of active transport mechanisms. Low temperatures applied to cellular systems are considered general inhibitors of metabolism and ATP generation, and hence, of active transport (Calatayud et al., 2011). The study of CYN transport at low temperatures shows differences with respect to the data obtained at 37° C only in secretory direction (B-A) (Figure 3), suggesting the possible involvement of a transcellular transport energy-dependent in this direction, a fact that corroborates the deductions derived from the efflux ratio analysis (section 3.1). However, the data do not support the presence of an active transport in absorptive direction, which could be involved in CYN transport but in a limited extent.

Chong et al (2002) suggested that CYN and bile acids share transport mechanisms since primary cells of murine liver showed lower cytotoxicity to CYN when co-exposed with bile acids, taurocholate and cholate. In the intestine, particularly in the apical membrane of the small intestine, there are several bile acid transporters: ASBT (SLC10A2) and different isoforms of OATP transporter family (Trauner and Boyer, 2002; Alrefai and Gill, 2007). The ASBT transporters participate in a sodium-dependent transport of bile salts (Dawson, 2011). The present study has revealed no influence of Na^+ ions in the transport

of CYN (section 3.5, data not shown), which could discard the participation of ASBT in the uptake of CYN through the apical domain.

Concerning OATPs, several isoforms have been described in the apical membrane of the intestinal epithelium: OATP1A2 or OATP-A and OATP2B1 or OATP-B (Roth et al., 2012). This family of carriers performs a transport ATP- and Na^+-independent (Roth et al., 2012). Some studies has reported the influence of GSH in the transport mediated by these carries. Physiologic GSH efflux stimulates OATP1-mediated substrate uptake into hepatocytes as well as into choroid plexus epithelial cells (Gao et al., 1999). Similar to OAPT1, OATP2-mediated taurocholate transport was stimulated by high intracellular GSH concentrations (Li et al., 2000). Furthermore, the optimum pH for the activity of OATP-B is around 5-5.5 (Kobayashi et al., 2003), is a H^+-dependent transporter. Our results show that this type of transport (independent of Na^+ and ATP, and dependent of H^+ and GSH) could participate in the transport of CYN from the apical side. However, the participation of this carrier was not evidenced in the assays performed with taurocholate, substrate with high affinity for OATPB (Hagenbuch and Meier, 2003), since the presence of this bile acid does not reduce the permeability of CYN at acid pHs.

Furthermore, Chong et al. (2002) showed that the protective effect of bile salts from CYN exposures was in a lesser extent than those observed for other toxins such as microcystin and lophyrotomin. Based on these results, the latter authors suggested that in addition to bile salts transporters, other mechanisms of transport of CYN may exist. It should be highlighted that CYN is a zwitterion and could enter the cell through the specific transporters for such molecules. The organic cation/zwitterion transporters (OCTNs) operate as uniporters which mediate facilitated diffusion or as Na^+/zwitterion cotransporters (Koepsell, 2013). Again, Na^+-dependent transport is discarded and the possibility of a facilitated transport mediated by OCTN rises. In the apical membrane of the intestinal epithelium the presence of various isoforms of OCTNs (OCTN1 and OCTN2) have been described, which mediate the transport of molecules such as L-

carnitine. The inhibition studies using this substrate do not reduce the A-B transport of CYN; therefore these carriers cannot be pointed as CYN transporters from the apical to basolateral side.

Summarising all the data obtained in the present work, CYN transport in absorptive (A-B) and secretory (B-A) directions using Caco-2 as a model of intestinal epithelium is reduced. Paracellular passive diffusion is predominant in apical-basolateral direction. In the secretory transport the involvement of active transport (P-glicoprotein, multidrug resistance proteins) seems to be likely, although an important role of the paracellular pathway have also been evidenced. The characteristics of the cell model, reduced paracellular space and high levels of expression of P-glycoprotein, may be in part the reason of the low transport observed from the apical to the basolateral side. Further studies could be desirable using models with greater pore diameter in the intercellular space and with an expression level of efflux proteins closer to that of the human intestine, such as co-cultures Caco-2/HT29-MTX (Hilgendorf et al., 2000) or monocultures of NCM-460 (Sahi et al., 1998). I*n vivo* assays are also needed to confirm the results derived from cell lines and to select the most suitable *in vitro* models for further studies.

Regarding the mechanisms of transport, in addition to the paracellular transport, a minor transcellular transport has been evidenced in the apical-basolateral direction which could be H^+ and GSH-dependent, facilitated (not dependent on energy) and Na^+-independent. The attempts made to target a specific transporter (bile salts or zwitterions carriers) have been unsuccessful, possibly due to the accentuated paracellular route. The identification of transporters responsible of the transcellular passage of CYN may require other approaches, such as the use of *Xenopus laevis* oocytes microinjected or overexpression of transporters in certain cell types.

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Figure legends

Figure 1. Apical-basolateral (A-B) (♦) and basolateral-apical (B-A) (■) transport of 0.8 mg/L of CYN in Caco-2 cells as a function of time. Values expressed as ng of CYN/mg protein (mean ± standard deviation, n = 3).

Figure 2. Apparent permeability coefficients ($P_{app,}$ 120 minutes) of CYN (0.8 mg/L) in apical-basolateral n (A-B) and basolateral-apical direction (B-A) in Caco-2 cells previously treated with EDTA (grey bars) or without previous treatment with EDTA (white bars). Values expressed as cm/s (mean ± standard deviation, n = 3). Significant differences with respect to the control (without EDTA) are marked with an asterisk (*) ($p < 0.05$).

Figure 3. Apparent permeability coefficients ($P_{app,}$ 120 minutes) of CYN (0.8 mg/L) in apical-basolateral (A-B) and basolateral-apical direction (B-A) in Caco-2 cells cultured at 37ºC (white bars) or 4 ºC (black bars). Values expressed as cm/s (mean ± standard deviation, n = 3). Significant differences of assays made at 4ºC with respect to those performed at 37ºC are marked with an asterisk (*) ($p < 0.05$).

Figure 4. Transport of CYN in the apical-basolateral direction in Caco-2 cells exposed to 0.8 mg/L of CYN over 120 minutes at various pHs. Values expressed as ng of CYN/mg protein (mean ± standard deviation, n = 3). Significant differences of assays made at pH apical/basolateral 5.5/7.2 with respect to the control (pH 7.2/7.2) are marked with an asterisk (*) ($p < 0.05$).

Figure 5. Transport of CYN in the apical-basolateral direction in Caco-2 cells exposed to 0.8 mg/L of CYN over 120 minutes in various conditions: CYN alone (white bar); co-exposure to CYN and 0.1 mM GSH (black bar); and co-exposure to CYN and 10 mM GSH (grey bar). Values expressed as ng of CYN/mg protein (mean ± standard deviation, n = 3). Significant differences with respect to the control (without GSH) are marked with an asterisk (*) ($p < 0.05$).

Figure 1

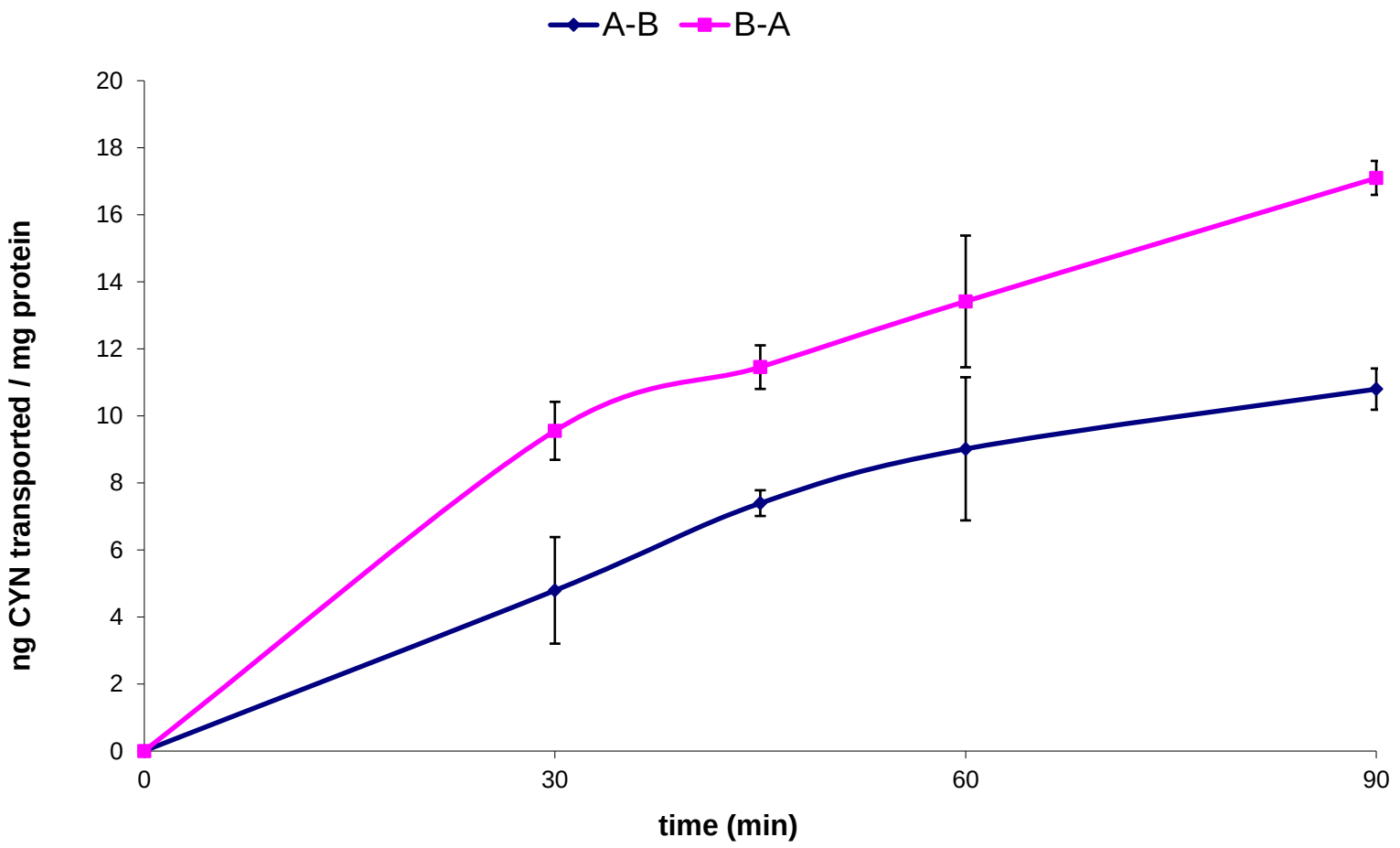

A-B
B-A
20
18
16
14
12
10
8
6
4
2
0
ng CYN transported / mg protein
0
30
60
90
time (min)

Figure 2

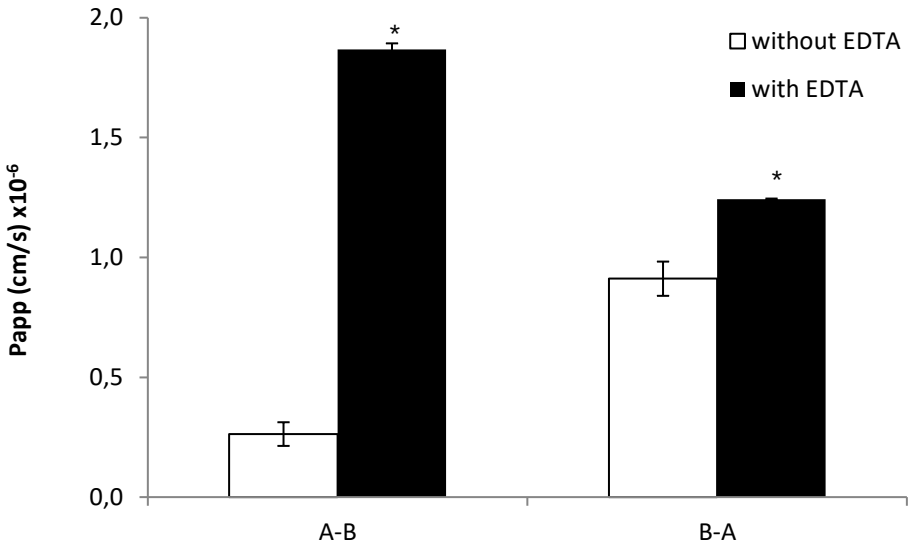


Figure 3

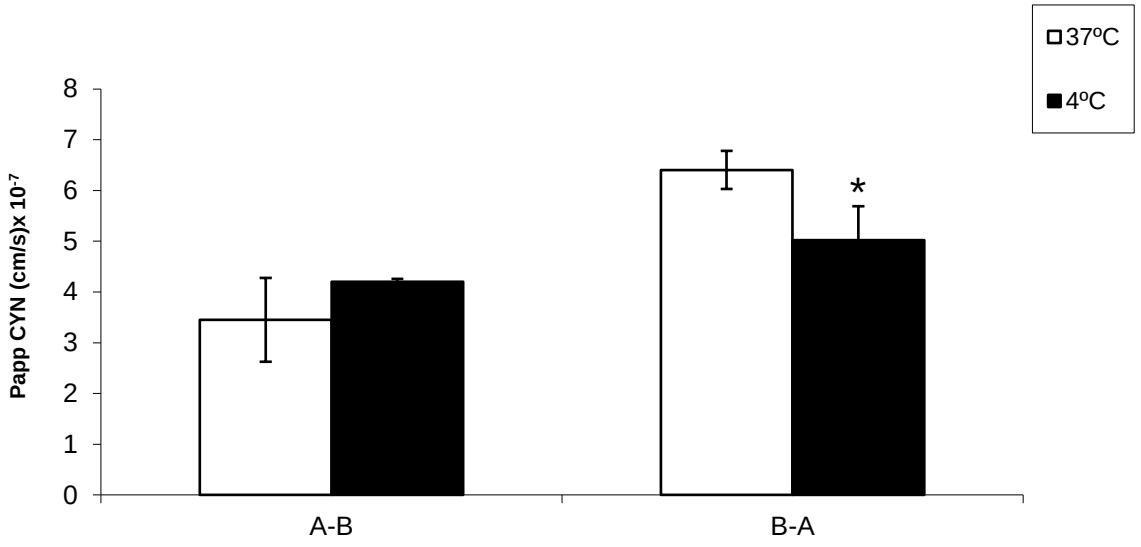


Figure 4

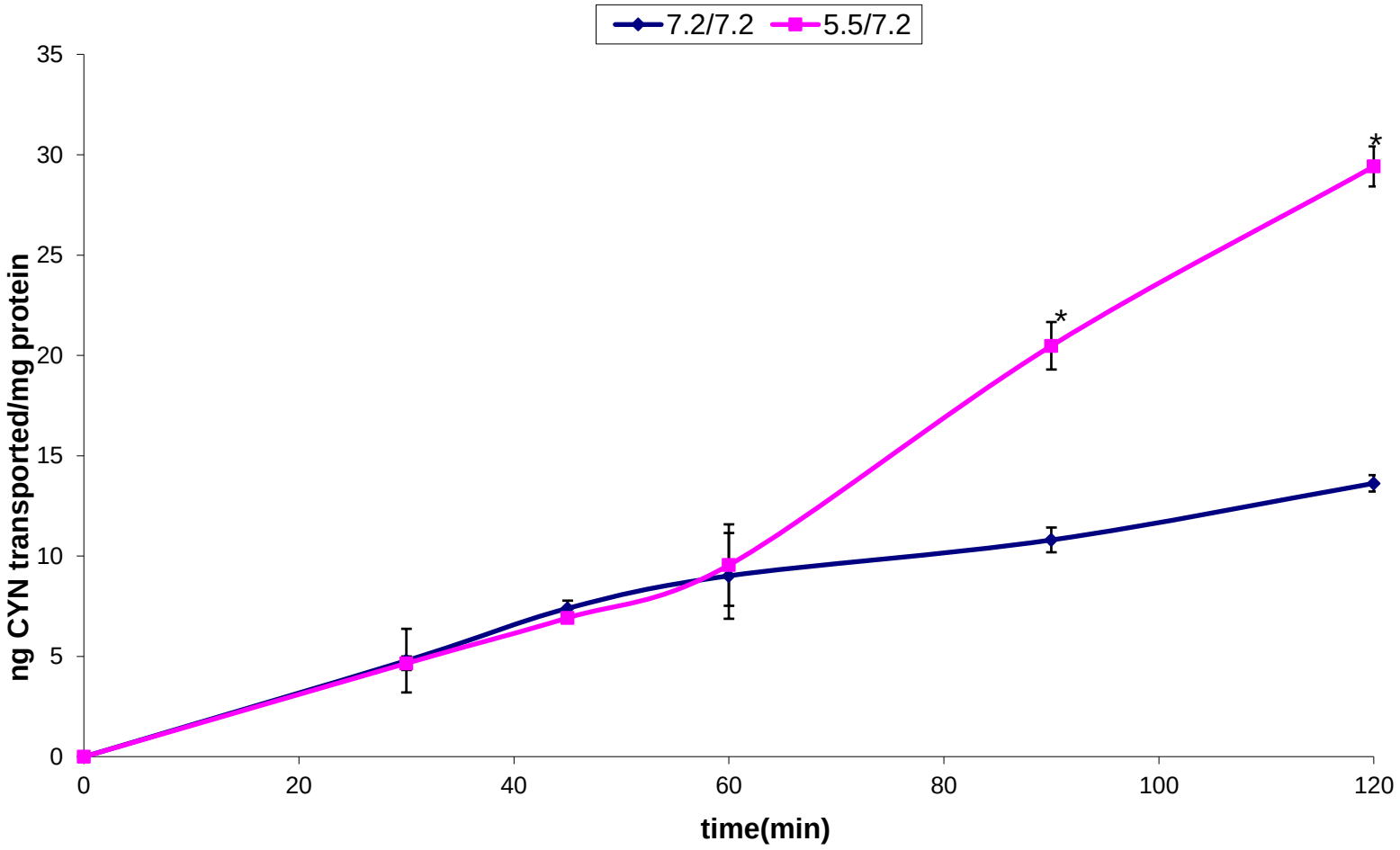
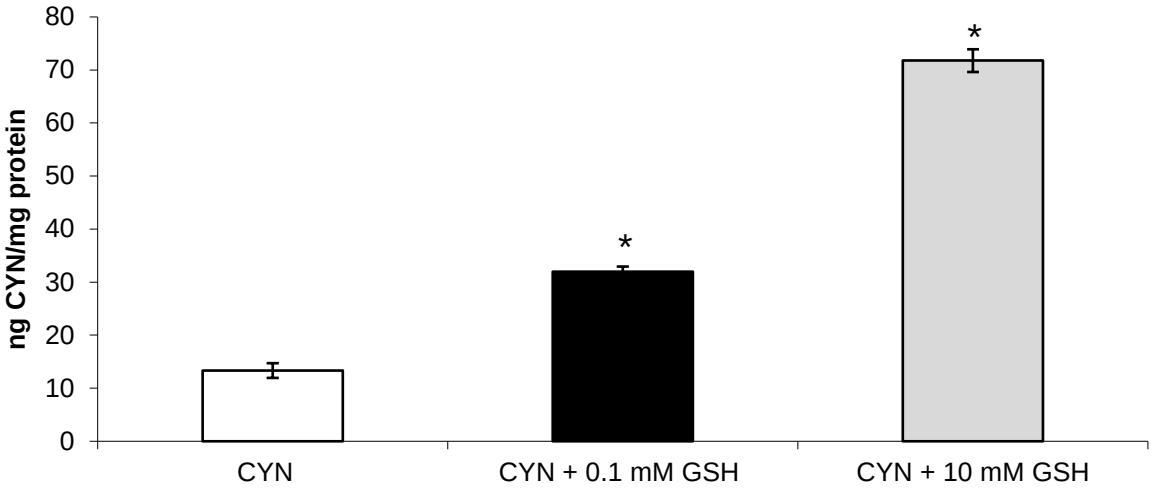

7.2/7.2
5.5/7.2
ng CYN transported/mg protein
time(min)
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
*
*

Figure 5


80
70
60
50
40
30
20
10
0
ng CYN/mg protein
*
*
CYN
CYN + 0.1 mM GSH
CYN + 10 mM GSH