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13-Desmethyl spirolide-c and 13,19-didesmethyl spirolide-c trans-epithelial permeabilities: Human intestinal permeability modelling

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ABSTRACT

Human intestinal permeability prediction is an increasingly important field that helps to explain how efficient the absorption of drugs is. Spirolides, cyclic imines produced by dinoflagellates from the genera *Alexandrium*, can be accumulated in mollusks usually consumed by humans. These compounds exert neurological symptoms when injected intra-peritoneally in mice, although they seem to be less toxic by oral administration. In this study, we evaluate two of the most abundant analogues, 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C and their ability to cross the human intestinal epithelium by the use of Caco-2 trans-epithelial permeability assays as a model. Toxin quantifications were carried out by using the liquid chromatography–tandem mass spectrometry analytical technique. We found that both compounds cross the Caco-2 epithelial barrier without altering the trans-epithelial electric resistance of the monolayer. The apparent permeability (P_{app}) coefficient calculated was $18.65 \pm 1.2 \times 10^{-6}$ cm/s for 13-desmethyl spirolide C while a little lesser, $12.32 \pm 3.18 \times 10^{-6}$ cm/s, for 13,19-didesmethyl spirolide C. P_{app} coefficients allow us to predict a human intestinal permeability ≥80% and ≥50%, respectively for each compound. Those results demonstrate that spirolides would be highly absorbed in the human intestine, thus being able to enter the circulatory system and to reach different organs where they could be accumulated or exert an unpredictable effect. Thus, it is necessary to carry out new studies about their pharmacokinetics and evaluate their potential acute and/or chronic effect on the human body.

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1. Introduction

Spirolides (SPX), cyclic imines produced by the dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum* (Cembella et al., 2000; Touzet et al., 2008) can be accumulated in molluscs consumed by humans. Those compounds mainly appeared in Europe and America mainly (Aasen et al., 2005; Alvarez et al., 2010; Amzil et al., 2007; Ciminiello et al., 2007; MacKinnon et al., 2006; Richard et al., 2001; Villar Gonzalez et al., 2006). SPXs were first found in 1995 during routine monitoring of polar lipophilic toxic compounds in mollusk extracts from aquaculture sites along the south-eastern coast of Nova Scotia in Canada. Hu et al. (1995) isolated four pure new compounds, SPXs A-D. However, 13-desmethyl spirolide-C (13-desme SPX-C) structure was not elucidated until 2001 when the same group isolated it from shellfish and from *A. ostenfeldii* cultures obtained in aquaculture site along the Atlantic coast of Nova Scotia (Hu et al., 2001). Another derivative, 13,19-didesmethyl spirolide-C (13,19-didesme SPX-C), was detected in two clonal isolates of *A. ostenfeldii* dinoflagellates from Limfjorden, Denmark (MacKinnon et al., 2006).

The molecular target of SPXs seems to be the acetylcholine receptors (AchRs). These compounds increase the mRNA levels of muscarinic and nicotinic AchRs and present a high affinity binding to them (Bourne et al., 2010; Gill et al., 2003; Vilarino et al., 2009). Recently Wandscheer et al. (2010) demonstrated that the human muscarinic AchRs are specific targets of the 13-desmethyl SPX-C.

When injected intra peritoneally to mice, SPXs exert neurological effects not fully characterized yet (Gill et al., 2003). *In vivo* toxicological studies demonstrated that these toxins induce neurological symptoms including stiffening and arching of the tail toward the head, tremors progressing to spam of the hind limbs, respiratory distress, tremors of the whole body and respiratory arrest (Gill et al., 2003).

Despite the fact that no toxic episode has been reported following human consumption of seafood contaminated with SPXs up to now, it is very important to evaluate their ability to cross the human intestinal epithelium to check their toxic potential when orally consumed.

Over the last few years the use of intestinal epithelial cell lines such as Caco-2 has dramatically increased in many research

Abbreviations: 13-DesmeSPX-C, 13-desmethyl spirolide-C; 13,19-DidesmeSPX-C, 13,19-didesmethyl spirolide-C; AchR, acetylcholine receptor; EMEM, Eagle's Modified Essential Medium; FBS, fetal bovine serum; HAB, harmful algal bloom; LC–MS/MS, liquid chromatography–tandem mass spectrometry; P_{app}, apparent permeability; SPXs, spirolides; TEER, trans-epithelial electrical resistance.

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fields usually to investigate whether a drug is actively or passively transported across the intestinal epithelium. Trans-epithelial permeability assays based on Caco-2, a cell line derived from a human colon adenocarcinoma, has been used extensively to model the human intestinal permeability to different drugs that reach the human intestine after oral consumption (Artursson et al., 2001; Lennernas, 2007).

In the present study we evaluate the ability of 13-desme SPX-C and 13,19-didesme SPX-C to cross the Caco-2 epithelium as a model to predict the human intestinal permeability to these toxins.

2. Materials and methods

2.1. Reagents

Eagle's Modified Essential Medium (EMEM) and foetal bovine serum were from PAA (Linz, Austria). 13-DesmeSPX-C and 13,19-didesmeSPX-C were from Laboratorio CIFGA S.A. (Lugo, Spain). Penicillin 10,000 U/mL/streptomycin 10,000 μg/mL and non-essential amino acids were from Biochrom AG (Berlin, Germany). Millicell culture plate inserts and Millicell Electrical Resistance System from Millipore (Madrid, Spain).

2.2. Caco-2 cells culture

Caco-2 cells from LGC Standards S.L.U. (Barcelona, Spain) were maintained at 37 °C in 95% relative humidity with 5% CO_2 in EMEM supplemented with 20% foetal bovine serum (FBS), 1% nonessential amino acids and penicillin 10,000 U/mL/streptomycin 10,000 μg/mL. For transport experiments, the cells were seeded onto the apical side (A) of Millicell culture plate inserts (diameter 1.1 cm^2, pore diameter 0.4 μm) at a target density of 6×10^4 viable cells cm^2. Cells of passage 20–60 were used throughout. The cells were allowed to grow and differentiate for 21–30 days before use. The cells were provided with 1.5 mL of supplemented EMEM on both the basolateral side (B) of the filter and with 0.5 mL on both the apical side.

2.3. Transepithelial permeability assays in Caco-2 cells

Once cells had spent 15–21 days seeded in the inserts the trans-epithelial electrical resistance (TEER) was measured with a Millipore Millicell Electrical Resistance System (ERS Millipore) after changing the medium. Control measurements from blank inserts were subtracted to calculate the TEER of the cell layer. Resistance values were multiplied by the surface area of the insert (1.1 cm^2) and expressed in ohms per centimetre squared. Caco-2 monolayers exhibiting a TEER value of more than 300 Ω cm^2 were used. The TEER was measured both in the beginning and at the end of the experiment. The transport experiments were performed in supplemented EMEM without FBS. Prior to assay, differentiated confluent Caco-2 monolayers on Millicell inserts were equilibrated in EMEM for 15 min. The transport experiment was started by positioning the inserts into clean wells containing 1.5 mL of fresh supplemented EMEM without FBS and adding a solution of 0.5 mL supplemented EMEM without FBS containing the toxin (13-desmeSPX-C or 13,19-didesmeSPX-C) or vehicle (methanol with 0.05% trifluoroacetic acid; control) to A of the cell monolayer.

Transport rates of 13-desmeSPX-C and 13,19-didesmeSPX-C were determined in A to B direction across the cell monolayer. Fifty microlitre samples were withdrawn from B at regular time intervals and immediately replaced by 50 μl of fresh supplemented EMEM without FBS. Samples were collected from A at the start and at the end of the experiment as well. The monolayers were incubated at 37 °C in 95% relative humidity with 5% CO_2. The samples taken from the transport experiments were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2.4. Calculation of permeability coefficient (P_{app})

The transported amount of each substrate across the monolayer was calculated by the value of the transported concentrations of the substrates multiplied by the volume, and apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{dQ/dt}{A \times C_0}$$

where dQ/dt, A and C_0 represent the amounts of the test substrates transported within a given time period, the surface area of monolayer and the initial amount of the substrates added to the apical side, respectively.

2.5. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

In order to know the toxin content, samples were analyzed by means of LC–MS/MS. The equipment used is a combination of HPLC plus mass detector. The HPLC system, from Shimadzu (Kyoto, Japan), consists of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and system controller (SCL-10Avp). This system is coupled to a QTRAP LC/MS/MS system from Applied Biosystems (USA), which integrate a hybrid quadrupole-linear ion trap mass spectrometer equipped with an ESI source. The nitrogen generator is a Nitrocraft NCLC/MS from Air Liquide (Spain). The column used for lipophilic toxins separations was a 2 mm × 50 mm BDS-Hypersil-C8 analytical column with a particle size of 3 μm and a 10 mm × 2.1 mm guard cartridge from Thermo (Waltham, MA, USA). The temperature was set at 25 °C. The composition of the mobile phase was: water (A) and acetonitrile/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30–90% B for 8 min, then, 90% B was hold for 3 min and reducing afterwards to 30% B over 0.5 min and hold for 5.5 min until the next run. The mobile phase flow rate was 0.2 mL/min and the injection volume was 5 μL. Collision-induced dissociation (CID) in the ion-trap MS was performed. The mass spectrometer was operated in multiple reactions monitoring (MRM) detecting in positive mode analyzing two product ions per compound. The transitions employed were: 13-desmethyl SPX-C (m/z 692.5 > 674.5, m/z 692.5 > 444.5) and 13,19-didesmethyl SPX-C (m/z 678.5 > 660.5, m/z 678.5 > 430.5). The electrospray ionization (ESI) source of QTRAP was operated with the following optimized values of source-dependent parameters: Curtain gasTM: 15 psi, collision-activated dissociation gas (CAD): 6 psi, IonSpray Voltage: 4000 V, temperature: 450 °C, gas 1: 50 psi and gas 2: 50 psi.

The estimation of each toxin concentration was determined in its own calibration curve. Calibrations were linear over the range 12.5–1000 ng/mL for 13-desmethyl spirolide C and 12.5–2000 ng/mL for 13,19-didesmethyl spirlide C using 9 points for each standard and the linear regression R^2 values were >0.999.

2.6. Statistical analysis

Results were analyzed using the Student's t-test for paired data where appropriate. A probability level of ≤0.05 was set to indicate statistical significance.

3. Results

Differentiated and polarized Caco-2 monolayers displaying TEER measurements ≥300 Ω cm^2 were used for the experiments. 1 μM 13-desmeSPX-C or 2 μM 13,19-didesmeSPX-C was added to the apical side of the inserts (A) and samples of 50 μL were collected after 3, 6 and 8 h of incubation from the basolateral side (B) and from both compartments at the end of each experiment, 10 h after the toxin addition in A (Fig. 1).

The detection of the toxins in the samples was carried out by LC–MS/MS. Chromatographic separation was performed in the conditions specified in Section 2. The transitions employed to look for the toxins were: m/z 692.5 > 674.5, m/z 692.5 > 444.5 for 13-desmethyl SPX-C and m/z 678.5 > 660.5, m/z 678.5 > 430.5 for 13,19-didesmethyl SPX-C (Fig. 2). Standard solutions of both toxins were used to generate calibration curves in the linear range between 12.5 and 1000 ng/mL for 13-desmethyl SPX-C and

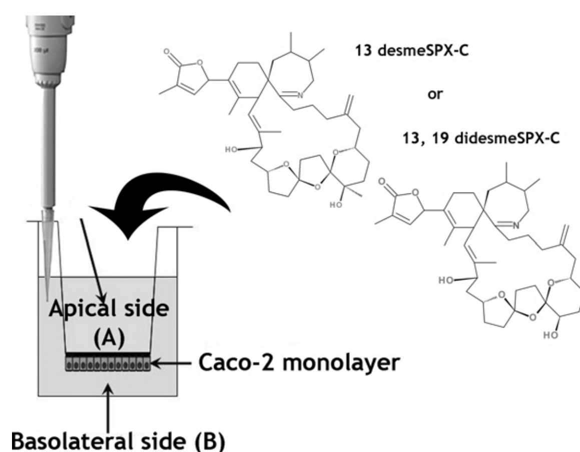

Fig. 1. Scheme of the experimental approach. 13-desmeSPX-C or 13,19-didesmeSPX-C or vehicle are added to the apical side of the inserts (A) and 50 μL samples are collected from the basolateral side (B) after 3, 6, 8 and 10 h of incubation. 50 μL samples are collected from A at the start and at the end points of the experiment as well. After, samples are analyzed and quantified for the presence of the 13-desmeSPX-C or 13,19-didesmeSPX-C.

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