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Océane Reale, Antoine Huguet, Valérie Fessard. Co-culture model of Caco-2/HT29-MTX cells: a promising tool for investigation of phycotoxins toxicity on the intestinal barrier. *Chemosphere*, 2021, 273, pp.128497. 10.1016/j.chemosphere.2020.128497 . anses-02958751

HAL Id: anses-02958751

<https://anses.hal.science/anses-02958751v1>

Submitted on 24 Apr 2023

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1 Article

2 Co-culture model of Caco-2/HT29-MTX cells: a 3 promising tool for investigation of phycotoxins 4 toxicity on the intestinal barrier

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10 Abstract:

11 Most lipophilic phycotoxins have been involved in human intoxications but some of these toxins
12 have never been proven to induce human gastro-intestinal symptoms, although intestinal damage
13 in rodents has been documented. For investigating the *in vitro* toxicological profile of lipophilic
14 phycotoxins on intestine, the epithelial Caco-2 cell line has been the most commonly used model.
15 Nevertheless, considering the complexity of the intestinal epithelium, *in vitro* co-cultures
16 integrating enterocyte-like and mucus-secreting cell types are expected to provide more relevant
17 data. In this study, the toxic effects (viability, inflammation, cellular monolayer integrity,
18 modulation of cell type proportion and production of mucus) of four lipophilic phycotoxins (PTX2,
19 YTX, AZA1 and OA) were evaluated in Caco-2/HT29-MTX co-cultured cells. The four toxins
20 induced a reduction of viability from 20% to 50% and affected the monolayer integrity. Our results
21 showed that the HT29-MTX cells population were more sensitive to OA and PTX2 than Caco-2
22 cells. Among the four phycotoxins, OA induced inflammation (28-fold increase of IL-8 release) and
23 also a slight increase of both mucus production (up-regulation of mucins mRNA expression) and
24 mucus secretion (mucus area and density). For PTX2 we observed an increase of IL-8 release but
25 weaker than OA. Intestinal cell models integrating several cell types can contribute to improve
26 hazard characterization and to describe more accurately the modes of action of phycotoxins.

27 **Keywords:** co-culture, Caco-2, HT29-MTX, phycotoxin, toxicity, intestine

28

29 1. Introduction

30 Phycotoxins, natural metabolites produced by unicellular micro-algae, can accumulate along the
31 marine food web, especially in filter-feeding bivalves (Reguera et al., 2014). The consumption of
32 contaminated seafood can provoke intoxications in humans depicted by various symptoms
33 (Valdiglesias et al., 2013; Khora and Jal, 2018). Among the lipophilic phycotoxins, azaspiracid-1
34 (AZA1) and okadaic acid (OA) are known to induce general weakness, nausea, diarrhea, abdominal
35 pains and vomiting (Twiner et al., 2008; Valdiglesias et al., 2013; Vilarino et al., 2018). In contrast, no
36 human intoxications have been proven with pectenotoxin 2 (PTX2) and yessotoxin (YTX) (EFSA,

37 2008, 2009; Dominguez et al., 2010), despite acute toxic effects described in rodents (Ishige et al.,
38 1988; Ogino et al., 1997; Aune et al., 2002; Callegari et al., 2006; Ito, 2006; Aasen et al., 2011).

39 The gastrointestinal epithelium acts as a barrier limiting the crossing of hazardous substances such
40 as phycotoxins, and consequently the potential toxicity to systemic organs. Nevertheless,
41 phycotoxins have been shown to induce some local toxicity in mice with macroscopical intestinal
42 damage such as cell detachment, fluid accumulation, villous erosion and dilatation of intestine tract
43 (Ishige et al., 1988; Ito et al., 2000; Ito et al., 2002; Ito et al., 2008; Aasen et al., 2010; Aune et al., 2012).
44 Besides, infiltration of immune cells in the lamina propria was reported in previous studies (Terao et
45 al., 1986; Aasen et al., 2011; Aune et al., 2012; Sosa et al., 2013; Kilcoyne et al., 2014).

46 If animal experiments remain crucial for addressing hazard characterization, design of *in vivo*
47 studies with phycotoxins is hampered due to the cost and the low availability of these substances.
48 Therefore, the use of human *in vitro* cell models is a good alternative. Apart from being simple, rapid
49 and reproducible, *in vitro* models enable using a broad range of conditions and studying the
50 mechanisms involved in the toxic response.

51 In intestinal Caco-2 epithelial cells, PTX2 inhibited F-actin polymerisation without affecting cell
52 viability, oxidative stress or cytokine IL-8 release (Ares et al., 2005; Alarcan et al., 2019).
53 Nevertheless, alteration of the intestinal monolayer integrity was pointed out with PTX2 in Caco-2
54 cells (Hess et al., 2007). In contrast, YTX induced a decrease of cell viability in various intestinal cell
55 lines but without any alteration of F-actin (Ares et al., 2005; Botana et al., 2014; Ferron et al., 2016).
56 Although no increase of oxidative stress and IL-8 release was observed in Caco-2 cells (Alarcan et al.,
57 2019), YTX affected the intestinal barrier integrity coupled with a degradation of E-cadherin
58 (Ronzitti et al., 2004; Hess et al., 2007). AZA1 triggered disturbance of junction proteins such as
59 occludin and E-cadherin (Abal et al., 2017), indicating an alteration of the intestinal barrier integrity
60 (Hess et al., 2007) even if no alteration of cell morphology was reported in Caco-2 cells (Ronzitti et
61 al., 2007; Abal et al., 2017). AZA1 induced also nucleus and mitochondrial damage, caspases
62 activation and autophagosomes formation in intestinal cells (Twiner et al., 2012; Abal et al., 2017;
63 Bodero et al., 2018). Concerning OA, studies reported a decrease of cell viability in various intestinal
64 cell lines (Blay and Poon, 1995; Ray et al., 2005; Serandour et al., 2012; Ferron et al., 2014; Hayashi et
65 al., 2018) but also activation of caspase-3 and NF- κ B as well as cell cycle disturbance (Ferron et al.,
66 2014). Besides, OA increased the paracellular permeability of intestinal cell monolayers (Tripuraneni
67 et al., 1997; Okada et al., 2000; Ehlers et al., 2011; Dietrich et al., 2019).

68 Surprisingly, the impact of phycotoxins on mucus secretion has never been investigated *in vitro*. In
69 fact, most of the *in vitro* studies have used only one cell line while the human intestinal epithelium is
70 composed of enterocytes (80-85 %) (Dutton et al., 2019), as well as various other cell types such as
71 endocrine cells, Paneth cells, stem cells, M cells and goblet cells (Berger et al., 2017). Several *in vitro*
72 human intestinal co-culture models combining enterocytes and mucus cells have been developed to
73 mimic the *in vivo* situation. Among them, the co-culture of intestinal Caco-2 enterocytes with
74 HT29-MTX goblet cells has shown a strong polarity with tight and adherens junctions, the presence
75 of a thick mucus layer and permeability values close to human intestine (Beduneau et al., 2014,
76 Ferraretto et al., 2018, Mahler et al., 2009, Pan et al., 2015). Nevertheless, such co-culture model has

77 never been used for investigating phycotoxins effects on the intestinal barrier. In this context, we
78 evaluated the toxicity of PTX2, YTX, AZA1 and OA on Caco-2/HT29-MTX co-cultures. After 21 days
79 of culture, the Caco-2 cell line differentiates into enterocyte-like cells (Natoli et al., 2012), and the
80 mucus secreting cell line HT29-MTX can mimic goblet cells (Martinez-Maqueda et al., 2015). Cell
81 viability, cellular monolayer integrity, modulation of cell type proportion, production of mucus and
82 inflammation were investigated with various methodologies such as transepithelial electrical
83 resistance (TEER), lucifer yellow (LY) crossing, ELISA, RT-qPCR, periodic acid Schiff and alcian blue
84 (PAS/AB) staining and High Content Analysis (HCA).

85

86 **2. Materials and Methods**

87 *2.1 Chemicals*

88 Cell culture products were purchased from Gibco (Cergy-Pontoise, France). Bovine serum albumin
89 (BSA), Tween 20, Triton, neutral red, LY and HEPES were supplied by Sigma-Aldrich (Saint Quentin
90 Fallavier, France). Monoclonal IL-8, biotinylated monoclonal IL-8 antibodies, SuperBlock blocking
91 buffer, streptavidin peroxidase, TNF α , TMB and paraformaldehyde were purchased from
92 ThermoFisher Scientific (Waltham, MA). For histology, ethanol, toluene, paraffin, Alcian Blue 8GX,
93 Schiff's reagent and Mayer Hematoxylin solution were purchased from VWR (Radnor, PA). Bluing
94 reagent was purchased from Microm (Brignais, France). Pectenotoxin-2, yessotoxin, azaspiracid-1
95 and okadaic acid, dissolved in methanol (MeOH), were purchased from the National Research
96 Council Canada (NRCC, Halifax, Nova Scotia, Canada).

97 *2.2 Cell culture and toxins exposure*

98 Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (ATCC) (Manassas,
99 VA), and Caco-2-GFP cells (transfected with EGFP-encoding lentivirus and stably expressing GFP)
100 were kindly provided by Dr. F. Barreau (INSERM IRSD U1220, Toulouse, France). Caco-2 and
101 Caco-2-GFP were maintained in Minimum Essential Medium containing 5.5 mM D-glucose, Earle's
102 salts and 2 mM L-alanyl-glutamine (MEM GlutaMAX) supplemented with 10% fetal bovine serum
103 (FBS), 1% non-essential amino acids, 50 IU/mL penicillin and 50 μ g/mL streptomycin at 37°C and 5%
104 CO $_2$. HT29-MTX were kindly provided by Dr T. Lesuffleur (INSERM U843, Paris, France) and
105 cultured in Dulbecco's Modified Eagle's Medium with high glucose (DMEM) supplemented with
106 10% inactivated FBS, 50 IU/mL penicillin and 50 μ g/mL streptomycin (complete DMEM) at 37°C and
107 5% CO $_2$. For subculture, cells were seeded in 75 cm 2 culture flasks and passaged once (for
108 HT29-MTX cells) or twice a week (for Caco-2 cells). For co-culture experiments, Caco-2 and
109 HT29-MTX cells (3:1) were seeded at 157,000 cells/cm 2 in complete DMEM. The ratio (3:1) was
110 selected as it is commonly used for mimicking the proportions found in human tissue (Kleiveland,
111 2015). 96-well plates were used for cytotoxicity, inflammation (IL-8 release) and
112 immunofluorescence assays, while 12-well and 24-well inserts (polyester membrane, 0.4 μ m pore
113 size; Corning, USA) were used for respectively qPCR assays, and for monolayer integrity and
114 histology assays respectively. For identification of the Caco-2 cells in the co-culture, Caco-2-GFP and
115 HT29-MTX cells (3:1) were seeded at 157,000 cells/cm 2 in complete DMEM in 96-well plates. Culture
116 medium was changed three times each week up to 21 days post-seeding. Differentiated co-cultured
117 cells were exposed to toxins in FBS free complete DMEM. A vehicle control (5% MeOH) was
118 included in each experiment.

119 *2.3 Neutral red uptake assay*

120 After 24 hrs of treatment, cytotoxicity was assessed by the neutral red uptake (NRU) assay as
121 previously described (Reale et al., 2019). Absorbance was measured at 540 nm with a microplate
122 reading spectrofluorometer (Fluostar OPTIMA, BMG Labtech, Champigny sur Marne, France).
123 Three independent experiments were performed, and for each experiment, the median of three
124 technical replicates was expressed relative to that of the vehicle control. The IC₅₀ was determined
125 using GraphPad Prism Software (version 5.0, GraphPad Software Inc., La Jolla, CA).

126 *2.4 Measurement of IL-8 release*

127 After 24 hrs of treatment, the medium of the 96-well plates used for the cytotoxicity assays was
128 collected for IL-8 measurement. The levels of interleukin-8 (IL-8) released in cell medium were
129 determined as previously described (Tarantini et al., 2015). TNF α (10 nM) was used as positive
130 control. Three independent experiments with three technical replicates per experiment were
131 performed.

132 *2.5 ZO-1 immunofluorescence*

133 After 24 hrs of treatment, the detection of ZO-1 was performed by immunofluorescence as
134 previously described (Ferron et al., 2014) with the following modifications. Antibodies used were
135 anti-rabbit ZO-1 (1/200, ThermoFisher Scientific) and goat anti-rabbit IgG (H+L) Alexa Fluor®647
136 (1/1000, ab150079, Abcam, Cambridge, UK). Cell identification was performed using DAPI
137 (Sigma-Aldrich) labelling. TNF α (50 nM) was used as positive control for a decrease of ZO-1
138 protein.. Two independent experiments with three technical replicates per experiment were
139 performed.

140 *2.6 Monolayer integrity assays*

141 *2.6.1 TEER measurement*

142 The trans-epithelial electrical resistance (TEER) of the Caco-2/HT29-MTX co-cultured cells was
143 measured to evaluate the monolayer integrity before and after a 24 hrs-treatment with toxins loaded
144 in the apical compartment. For the TEER measurement, assessed with a Millicell Electrical
145 Resistance System supplied by Millipore (Merck, Burlington, MA), the Caco-2/HT29-MTX
146 co-cultured cells were incubated for 30 min at 37°C with 300 and 700 μ L transport buffer (HBSS,
147 HEPES 5 mM, pH 7.4) respectively in the apical and basolateral compartments. For each
148 experimental condition, cell-free inserts were included. The TEER value was expressed as ohm.cm²
149 and calculated according to the following equation:

$$150 \quad \text{TEER} = (R_s - R_b) \times A$$

151 with R_s the resistance value of the Caco-2/HT29-MTX cells monolayer on inserts, R_b the resistance
152 value of the cell-free inserts, and A the insert area (0.33 cm²). Based on our lab historical data, only
153 Caco-2/HT29-MTX cells monolayer with a TEER > 130 ohm.cm² were considered suitable
154 corresponding to a permeability coefficient P_{app} below 1%/h. Two independent experiments were
155 performed.

156 *2.6.2 LY crossing*

157 The impact of toxins on the Caco-2/HT29-MTX cells monolayer integrity was also evaluated by the
158 ability of LY, a paracellular marker, to cross the Caco-2/HT29-MTX cells monolayer. Following the
159 second TEER measurement, 100 μ L of 1 mM LY in transport buffer was loaded in the apical
160 compartment, and the basolateral compartment was filled with 500 μ L of transport buffer. The
161 plates were incubated at 37 °C and shaken at 100 rpm on a microplate shaker (Thermo Scientific) for
162 3 hrs. The basolateral compartment was harvested and fluorescence was measured (excitation at 405
163 nm, emission at 520 nm) with a microplate reading spectrofluorometer (Fluostar OPTIMA). The
164 amount of LY crossed into the basolateral compartment was expressed relative to that of the initial

165 amount loaded into the apical compartment, and then expressed per hour. Two independent
166 experiments were performed. A standard curve of LY from 1 μ M to 0.01 μ M was included.

167 2.7 *Mucus analysis*

168 Following 24 hrs treatment, inserts with Caco-2/HT29-MTX co-cultured cells were washed twice
169 with PBS before removing the membranes from the inserts. After placement in embedding cassettes,
170 membranes were fixed with paraformaldehyde 4% in distilled water for 60 min at room
171 temperature. Dehydration and paraffin embedding of the membranes were performed using an
172 automate (Shandon Cidabelle 100, GMI, Ramsey, MN) as follow: three washes in PBS, ethanol (70%
173 1 hr, 96% 3 x 2 hrs, and 100% 3 x 2 hrs), toluene (2 x 2 hrs), paraffin (2 x 2 hrs). Histological sections of
174 4 μ m thickness were cut using a Shandon Finesse E+ microtome (ThermoScientific). Slides were
175 dewaxed and soaked in xylene (Sigma-Aldrich, Saint Quentin Fallavier, France) for 15 min and then
176 in ethanol 100° for 10 min with an automate LEICA ST 5020 (Leica, Wetzlar, Germany). After
177 staining, images were acquired with a scanner NanooZommer (50 \times magnification) and analysed
178 with NIS-Element AR Analysis 4.11.00 software. Two independent experiments were performed
179 and, for each independent experiment, the median of three sections was calculated. Slides were
180 coded for blind scoring.

181 2.7.1 *AB staining*

182 Alcian blue (AB) staining was used to visualize acidic mucins. Acidic mucins were stained with 0.1%
183 AB 8GX in 3% glacial acetic acid pH 2.6 for 20 min. After wash with distilled water, nuclei were
184 stained with 0.1% nuclear fast red solution in distilled water for 5 min. Slides were dehydrated in
185 ethanol 100° for 6 min and then in xylene for 4 min.

186 2.7.2 *PAS staining*

187 Periodic Acid Schiff's (PAS) staining was used to visualize neutral mucins. Slides were stained with
188 1% PAS in distilled water for 5 min, in Schiff's reagent for 12 min and then stained with 10% Mayer
189 Hematoxylin solution and with Bluing reagent for 1.5 min. Slides were dehydrated in ethanol 100°
190 for 6 min and then in xylene for 4 min.

191 2.8 *RT-qPCR*

192 For each toxin, maximal applied concentrations were chosen as IC₁₅ according to the NRU assay
193 results. After 24 hrs of treatment with PTX2 (100, 150, and 200 nM), YTX (25, 50, and 100 nM), AZA1
194 (7.5, 15, and 30 nM) and OA (75, 125, and 175 nM), experiments were performed as previously
195 described (Reale et al., 2019) with the following modifications. Reverse transcription was performed
196 with 2.5 μ g of total RNA, and quantitative PCR reactions were carried out with 6.25, 1.56 or 0.39 ng
197 cDNA, depending of the target gene. All primers were purchased from Sigma-Aldrich, and
198 additionnal informations on target genes and oligonucleotide primers are listed in Table S1.
199 Calibration curves were established for each gene from a serial two-fold dilution of a reference
200 sample (pool of cDNA samples). According to these calibration curves, for each sample, mean
201 relative amounts of mRNA of the targeted genes were calculated and then normalized to that of the
202 reference gene. Using NormFinder software, the gene *RPLP0* was chosen as a reference gene since it
203 did not exhibit any significant variation of expression among the samples. The normalized means
204 were used for statistical analyses and values were presented as arbitrary units. Three independent
205 experiments were performed.

206 2.9 *Identification of the Caco-2 cells population in the Caco-2/HT29-MTX co-culture*

207 As PTX2 and OA showed interesting results, we evaluated which cell subpopulation (Caco-2 or
208 HT29-MTX) was affected. After 24 hrs of toxin treatment, Caco-2-GFP/HT29-MTX cells (3:1) were
209 co-incubated at 37 °C for 15 min with 3 μ g/mL Hoescht 33342 (Sigma-Aldrich) in complete DMEM.

210 Fluorescence was monitored with an Arrayscan VTI HCS Reader (Thermo Scientific) associated with
 211 a live cell chamber. The Target Activation module of the BioApplication software was used to
 212 identify Caco-2-GFP cells. Total cell count was performed using Hoescht labelling. Wells with only
 213 Caco-2-GFP cells was used as positive control. Three independent experiments were performed, and
 214 for each experimental condition, the number of Caco-2-GFP cells was expressed relative to the total
 215 cell number.

216 2.10 Statistics

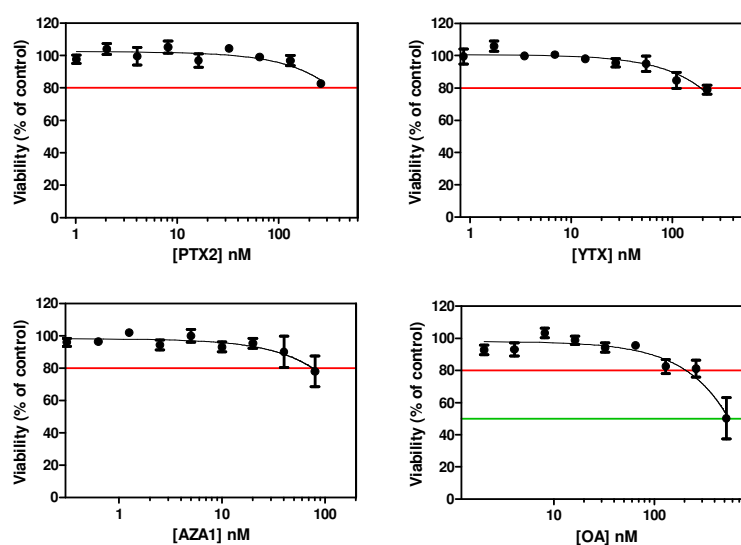
217 GraphPad Prism software was used for statistical analyses. An analysis of variance (ANOVA) was
 218 performed, and, when the effect of concentration was significant ($P < 0.05$), the values were
 219 compared to the control using the Dunnett's test. Differences were declared significant at $P < 0.05$.
 220 The values presented are means \pm SEM.

221 3. Results

222

223 3.1. Cytotoxic and morphological effects of phycotoxins

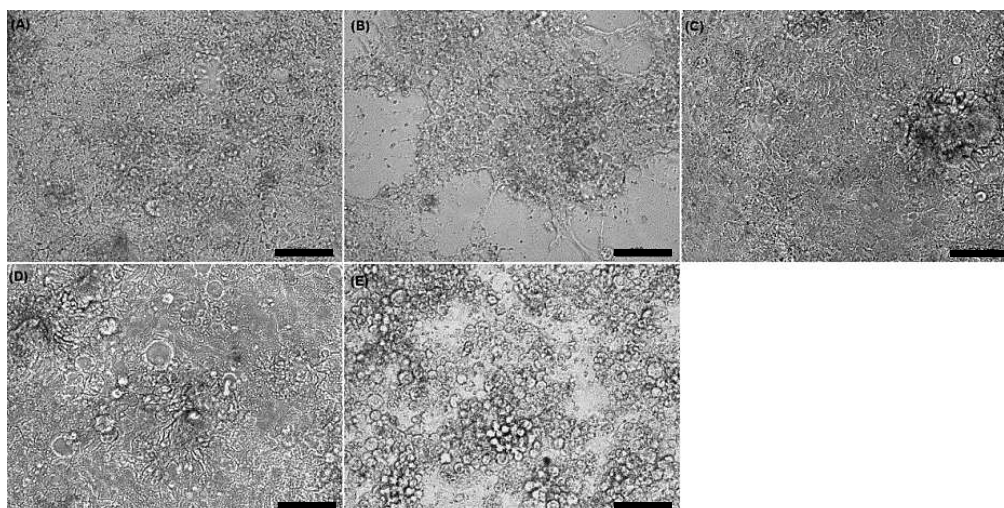
224 After a 24 hrs treatment, an IC_{50} could only be established for OA ($IC_{50} = 550 \pm 104$ nM) (Figure 1). A
 225 slight decrease of cell viability (around 20%) was observed with the highest concentrations for the
 226 other toxins (260 nM PTX2, 200 nM YTX and 77 nM AZA1).



227

228 **Figure 1. Cytotoxicity on Caco-2/HT29-MTX co-cultured cells after a 24 hrs exposure to PTX2, YTX,**
 229 **AZA1 and OA in 96-well plates.** Cytotoxicity was measured by the neutral red uptake (NRU) assay.
 230 Red line: 80% of viability; green line: 50% of viability. Values are presented as means \pm SEM, and
 231 expressed as percentages relative to the vehicle control. Three independent experiments with three
 232 technical replicates per experiment were performed.

233 Cellular morphology was observed after 24 hrs of treatment (Figure 2). While YTX and AZA1 did
 234 not induce any morphological changes, treatment with PTX2 and OA showed large areas without
 235 cells (above 65 nM PTX2) as well as severe cell rounding (above 130 nM OA).



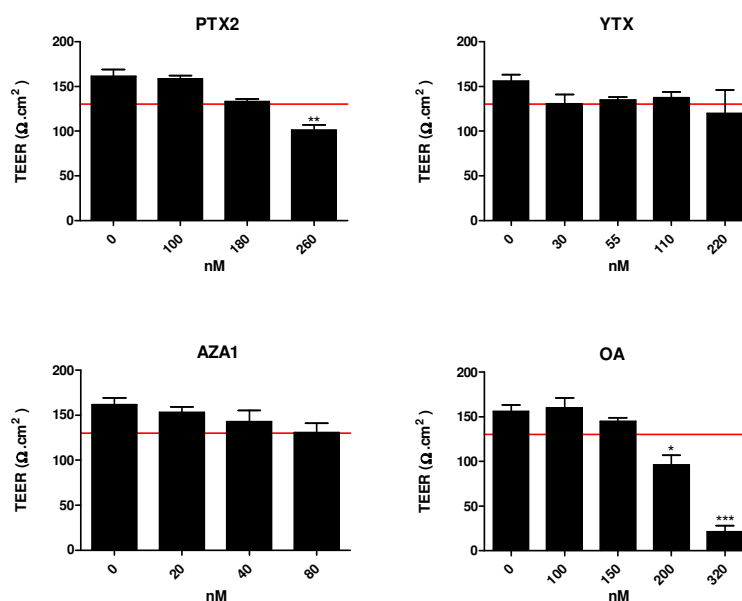
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237 **Figure 2. Morphological changes of Caco-2/HT29-MTX co-cultured cells after 24 hrs exposure to**
 238 **PTX2, YTX, AZA1 and OA in 96-well plates.** Evaluation of cell morphology was carried out by
 239 phase contrast microscopy. Each image is representative of three independent experiments. (A):
 240 vehicle control (5% MeOH), (B): 260 nM PTX2, (C): 220 nM YTX, (D): 80 nM AZA1, and (E): 520 nM
 241 OA. Scale bar = 100 μm .

242 3.2. Cellular monolayer integrity

243 3.2.1 TEER and LY permeability

244 The TEER value for the vehicle control was on average $162 \pm 7.5 \text{ ohm.cm}^2$ (Figure 3). The
 245 integrity of the Caco-2/HT29-MTX cells monolayer was affected after 24 hrs treatment with
 246 PTX2 and OA. TEER values decreased significantly reaching -38% with 260 nM PTX2, and -87%
 247 with 320 nM OA. A slight but not significant decrease was noticed after exposure to YTX and
 248 AZA1.

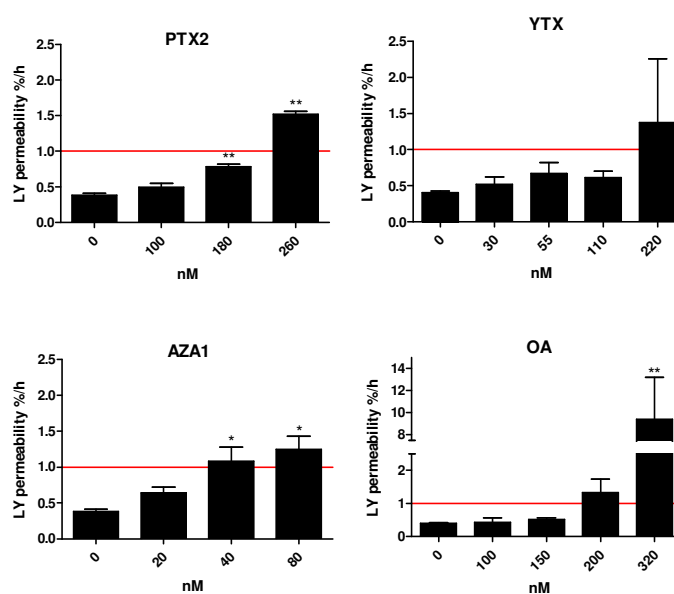


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250 **Figure 3. TEER measurement on Caco-2/HT29-MTX co-cultured cells after 24 hrs exposure to**
 251 **PTX2, YTX, AZA1 and OA in 24-well inserts.** Red line: 130 ohm.cm^2 . Values are presented as means

252 \pm SEM. Two independent experiments with two technical replicates per experiment were performed.
 253 *, **, ***: value significantly different from the vehicle control (respectively $P < 0.05$, $P < 0.01$ and $P <$
 254 0.001).

255 For the vehicle control, the relative amount of LY that crossed into the basolateral compartment
 256 was on average 0.400 ± 0.005 %/h (Figure 4). The relative amount of LY crossing the
 257 Caco-2/HT29-MTX cells monolayer was significantly increased reaching 9.4, 1.5 and 1.3 %/h at
 258 the highest concentration for OA, PTX2 and AZA1 respectively. For YTX, it was difficult to
 259 clearly state to an increase of LY crossing due the variability observed at the highest
 260 concentration.



261

262

263 **Figure 4. LY crossing through Caco-2/HT29-MTX co-cultured cells after 24 hrs exposure to PTX2,**
 264 **YTX, AZA1 and OA in 24-well inserts.** Values are presented as means \pm SEM, and expressed as
 265 percentage of LY that had crossed into the basolateral compartment per hour. Two independent
 266 experiments with two technical replicates per experiment were performed. *, **: value significantly
 267 different from the vehicle control (respectively $P < 0.01$ and $P < 0.001$).

268 3.2.2 ZO-1 immunofluorescence

269 In the vehicle control, ZO-1 was distributed mainly at the cell border (Figure 5). Irrespective of
 270 the toxin, ZO-1 localization was perturbed and redistributed, showing irregular cell borders
 271 labelling due to ZO-1 transfer from tight junctions to the cytoplasm. However, these effects
 272 were less pronounced with YTX and AZA1. As observed with light microscopy, 260 nM PTX2
 273 and 320 nM OA showed areas without cells.

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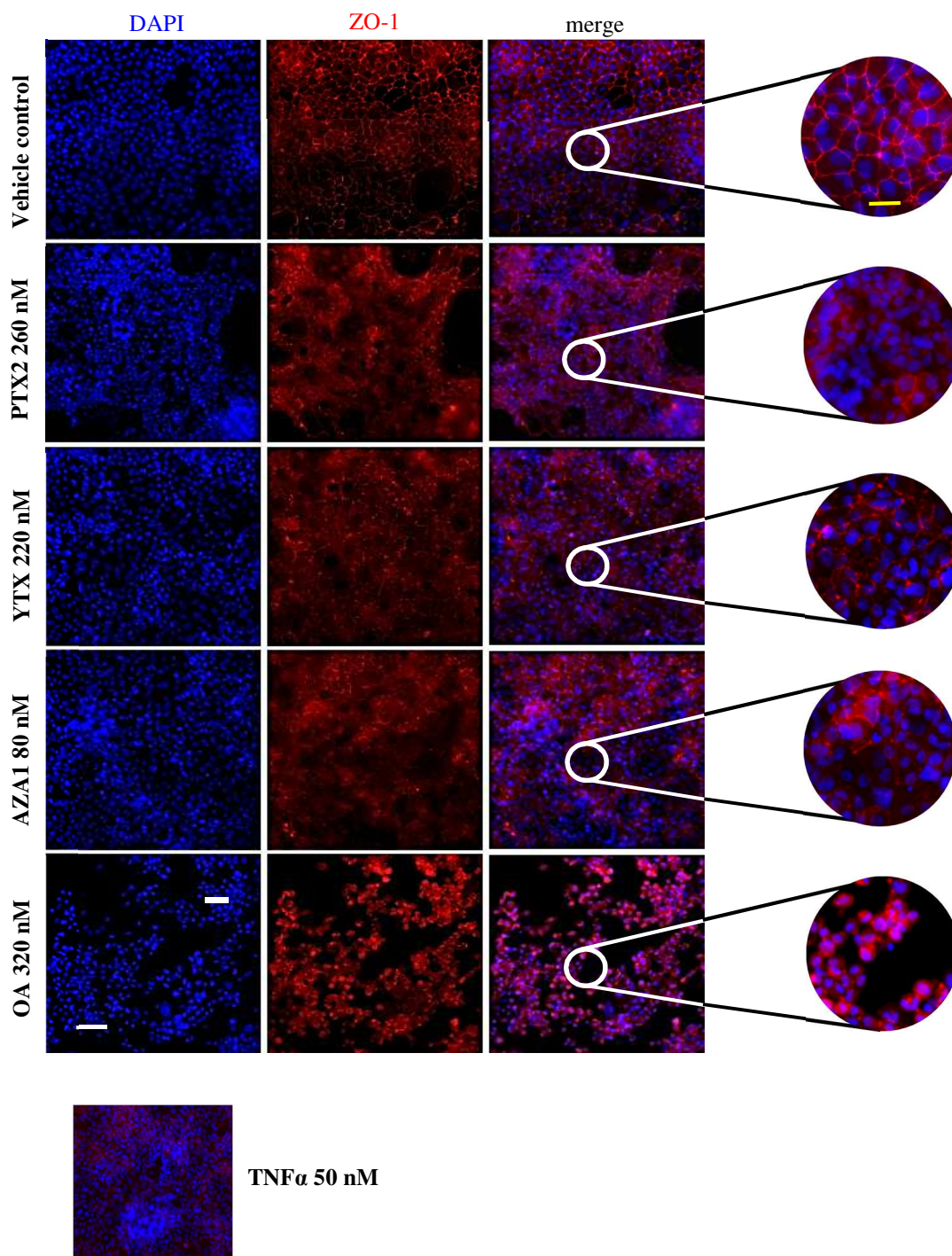
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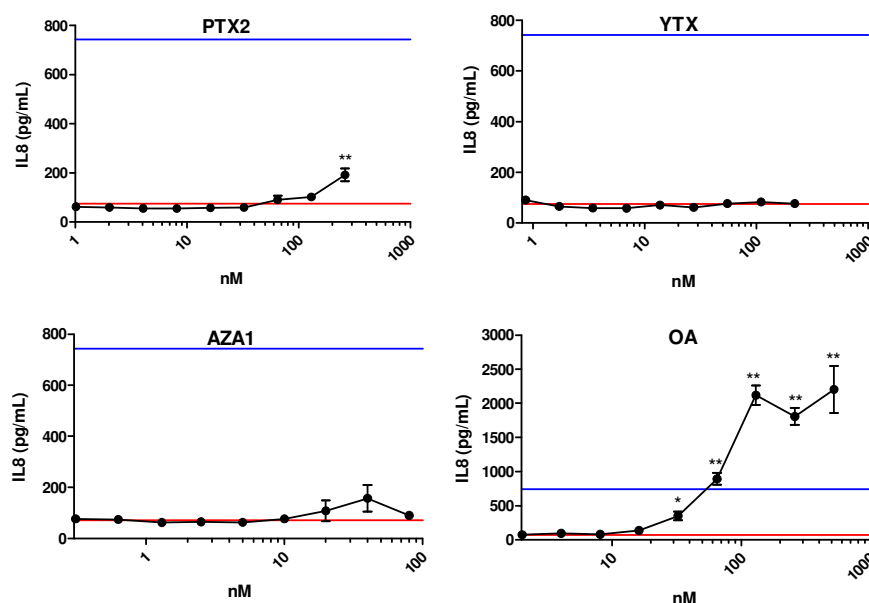
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Figure 5. Immunofluorescence localization of zonula occludens (ZO-1) in Caco-2/HT29-MTX co-cultured cells after 24 hrs exposure to PTX2, YTX, AZA1 and OA in 96-well plates. 50 nMTNF α was used as positive control. ZO-1 (red) and DAPI (blue) nucleus staining were carried out by HCA. Each image is representative of two independent experiments. Scale bar = 60 μ m (white bar) and 3 μ m (yellow bar).

307 3.3. Inflammatory response

308 In the vehicle control, the concentration of IL-8 released in the cell medium was on average 73.2 ± 3.3
 309 pg/mL (Figure 6). A concentration dependent increase of IL-8 release was observed after 24 hrs
 310 exposure to PTX2 and OA. Compared to the vehicle control, the IL-8 concentration increased
 311 significantly up to 3-fold with 260 nM PTX2 and 28-fold with 520 nM OA. No significant
 312 modification was pointed out following treatment with YTX up to 220 nM. AZA1 induced a slight
 313 but non significant increase of IL-8 release at 40 nM that was no longer observed at the highest
 314 concentration (80 nM).



315 **Figure 6. IL-8 release in Caco-2/HT29-MTX co-cultured cells after 24 hrs exposure to PTX2, YTX,**
 316 **AZA1 and OA in 96-well plates.** Red line: IL-8 release concentration in the vehicle control (73
 317 pg/mL). Values are presented as means \pm SEM. Three independent experiments with three technical
 318 replicates per experiment were performed. *, **: value significantly different from the vehicle control
 319 (respectively $P < 0.05$ and $P < 0.001$). IL-8 release of the TNF α positive control was 742 ± 7.5 pg/mL
 320 (blue line).
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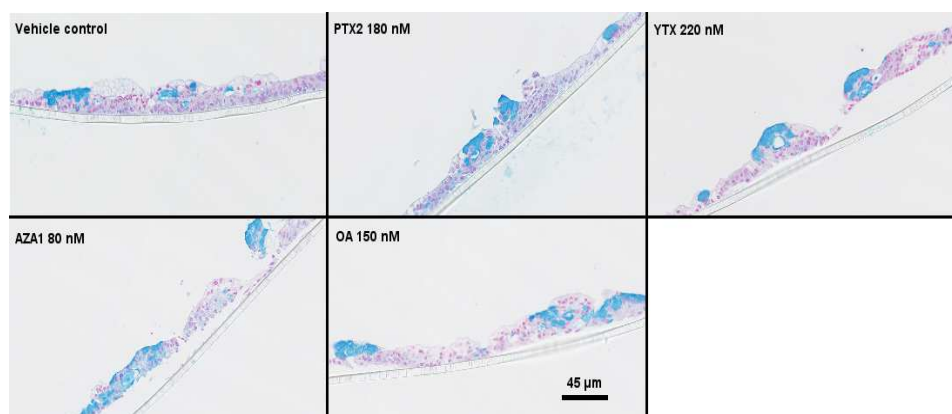
322 3.4. Mucus layer

323 3.4.1 Acidic mucins

324 Figure 7A presents the observations by light microscopy of transversal sections of
 325 Caco-2/HT29-MTX co-cultures following AB staining. Figure 7B presents the percentage area of AB
 326 staining, and the mean intensity of all analyzed blue pixels. PTX2 did not show any significant
 327 modification of both acidic mucus area and intensity compared to the vehicle control. YTX, AZA1
 328 and OA induced an increase of AB area of 1.5-, 1.7- and 1.4-fold respectively, although not
 329 statistically significant. The increase of mucus area following AZA1 exposure was concomitant with
 330 a significant decrease (18%) of AB mean intensity. The two other toxins, YTX and OA, did not affect
 331 AB intensity.

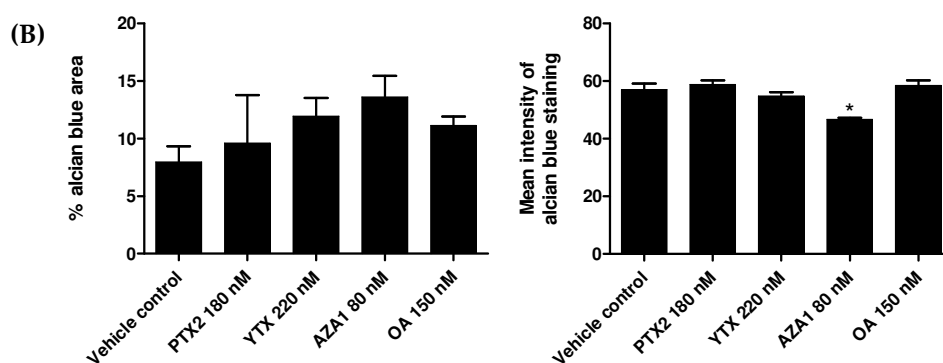
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Figure 7. Staining of acidic mucins in Caco-2/HT29-MTX co-cultured cells with alcian blue after 24 hrs exposure to PTX2, YTX, AZA1 and OA in 24-well inserts. (A) Observation by light microscopy of transversal sections. Scale bar = 45 μ m. (B) Percentage area of alcian blue staining and mean intensity of all analyzed blue pixels. Values are presented as means \pm SEM. Two independent experiments with one technical replicates per experiment were performed. *: value significantly different from the vehicle control ($P < 0.05$).

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3.4.2 Neutral mucins

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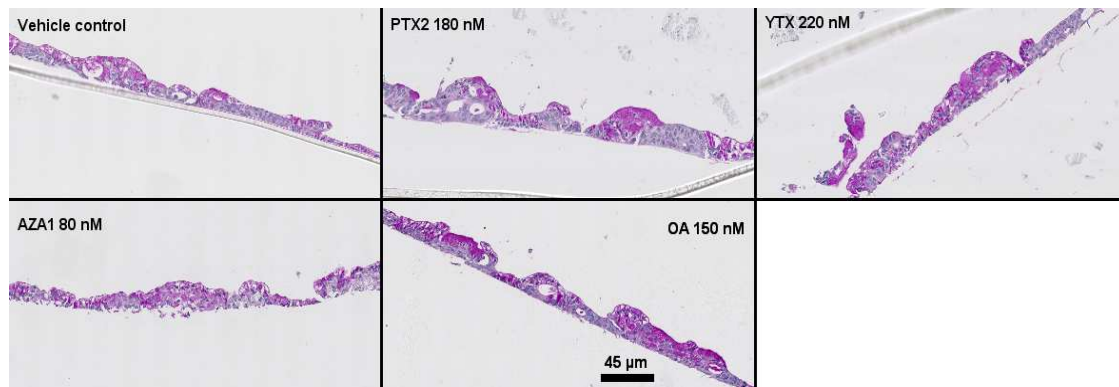
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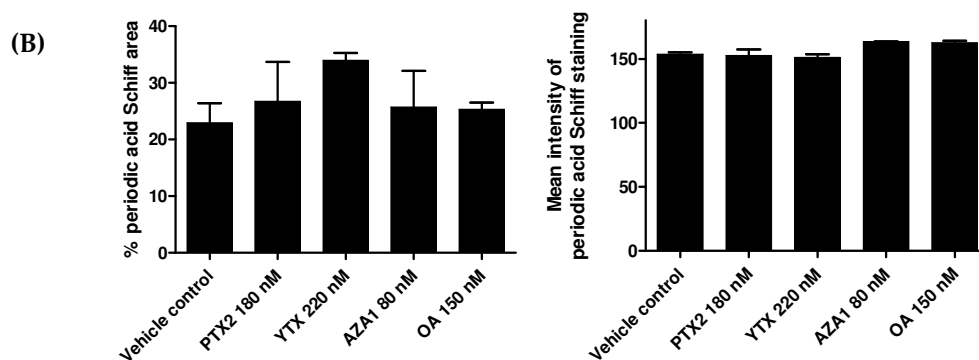
Figure 8A presents the observations by light microscopy of transversal sections of Caco-2/HT29-MTX co-cultures following PAS staining. Figure 8B presents the percentage area of PAS staining, and the mean intensity of all analyzed purple pixels. PTX2 did not show any significant modification of both neutral mucus area and intensity compared to the vehicle control. YTX exposure resulted in an increase of PAS staining area although not statistically significant and without any change of the intensity. A slight, but non-significant, increase of PAS mucus area was observed for OA and AZA1.

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(A)



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353 **Figure 8. Staining of neutral mucins in Caco-2/HT29-MTX co-cultured cells with periodic acid**
 354 **Schiff after 24 hrs exposure to PTX2, YTX, AZA1 and OA in 24-well inserts. (A) Observation by**
 355 **light microscopy of transversal sections. Scale bar = 45 μ m. (B) Percentage area of periodic acid Schiff**
 356 **staining and mean intensity of all analyzed purple pixels. Values are presented as means \pm SEM. Two**
 357 **independent experiments with one technical replicates per experiment were performed**

358 3.5. Modulation of gene expression

359 Additional investigation was done by analyzing some key genes involved in inflammation,
 360 adherens and tight junctions, mucus production and channels (Table 1). IC_{15} of each toxin was
 361 chosen as maximal concentration. For inflammation, *IL-8* was up regulated for AZA1 (2.9-fold at 15
 362 nM) although not statistically significant. A concentration-dependent up regulation of *IL-8* was
 363 observed with OA reaching 7.9-fold at 175 nM. Concerning claudin genes related to adhesion, a
 364 concentration-dependent up regulation was observed with 200 nM PTX2 for *CLDN3* (1.6-fold), and
 365 also for *CLDN4* (2.8-fold) but not statistically significant. *CLDN4* was also up regulated, but not
 366 significantly, with 30 nM AZA1 (2.9-fold) and 175 nM OA (2.5-fold). For mucus, gene expressions of
 367 *MUC12* and *MUC13* were significantly up regulated respectively by 200 nM PTX2 (1.7-fold) and 175
 368 nM OA (2.1-fold). A gene implicated in lipid droplet structure (*PLIN2*) was up regulated with OA
 369 (1.8-fold at 125 nM) and AZA1 (1.7-fold at 30 nM). This up regulation was concentration-dependent.
 370 Except for PTX2, all the toxins induced a significant down regulation of *TFF3* expression ranging
 371 from 0.3-fold to 0.5-fold inhibition, the encoded protein being involved in mucosal protection and
 372 stabilization of the mucus layer. Regarding channels expression, *AQP3* was significantly down
 373 regulated ranging from 0.4-fold to 0.6-fold inhibition in a concentration dependent-manner with all
 374 the toxins.

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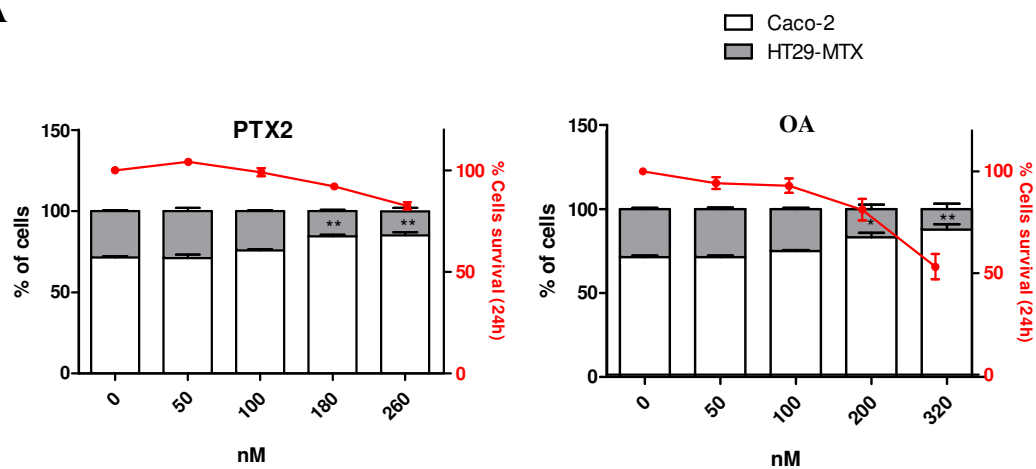
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389 *3.6 Evolution of the Caco-2-GFP/HT29-MTX proportion following treatment with PTX2 and OA*

390 After 21 days post-seeding, the Caco-2-GFP/HT29-MTX proportion in the vehicle control was on
 391 average 71:29 (Figure 9A). The HT29-MTX cells were mainly aggregated in islets (Figure 9B). The
 392 percentage of Caco-2-GFP cells increased significantly after exposure to the toxins, the
 393 Caco-2-GFP/HT29-MTX proportion reached 85:15 and 88:12 with 260 nM PTX2 and 320 nM OA
 394 respectively concomitantly with a decrease of cell viability. PTX2 treatment provoked the formation
 395 of area without cells from 180 nM. Such effects, but weaker, were also observed with OA from 200
 396 nM.

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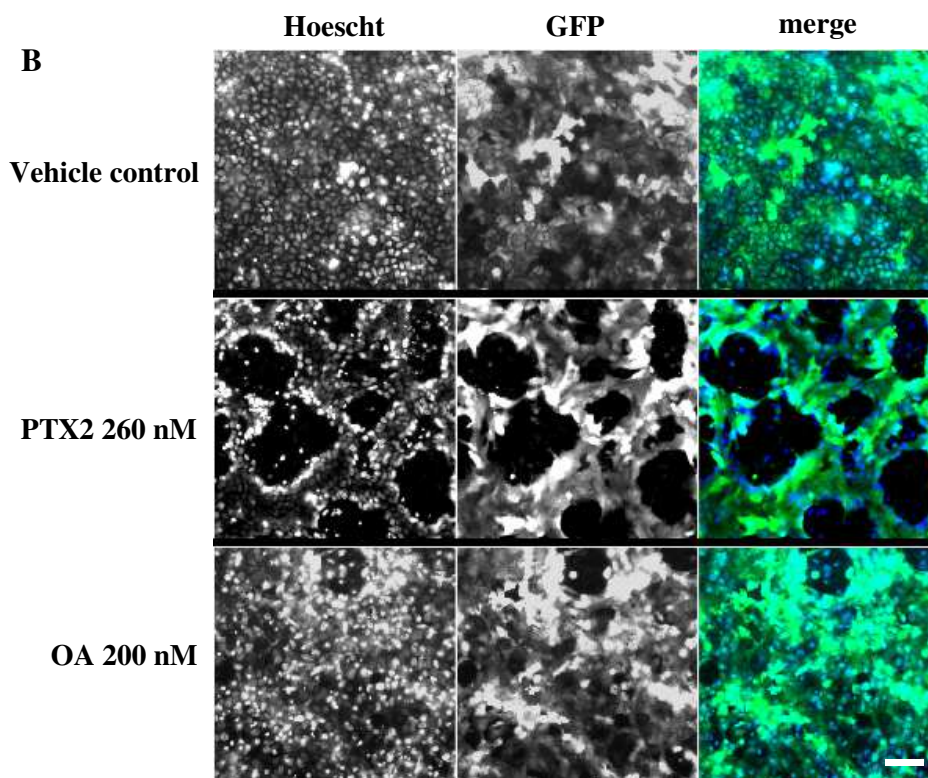
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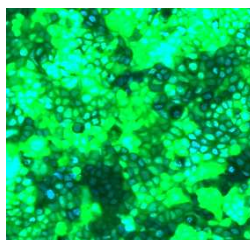
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Positive control
Caco-2-GFP



419 **Figure 9. Percentage of Caco-2-GFP and HT29-MTX cells co-cultured after 24 hrs exposure to PTX2 and**
420 **OA in 96-well plates.** Caco-2-GFP cells were identified as green fluorescent cells, and total cell count was
421 determined using Hoescht 33342 labelling. Percentages of each cell line are expressed relative to the total
422 cell number and presented as means \pm SEM. Positive control of the full fluorescence represents only
423 Caco-2-GFP. Three independent experiments were performed. *, **: value significantly different from the
424 vehicle control (respectively $P < 0.01$ and $P < 0.001$). Scale bar = 60 μ m.

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4. Discussion

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The Caco-2 and HT29-MTX cell lines represent the two major cell types (absorptive and goblet cells respectively) found in the small intestinal epithelium. In our study, the Caco-2/HT29-MTX cellular monolayer presented a TEER value (around 160 ohm.cm²) much lower than the Caco-2 cells monolayer (400-800 ohm.cm² depending on the cell culture method) (Tor, 2015), but closer to the value measured in human intestine (approximately 40 ohm.cm²) (Ferrec et al., 2001; Takenaka et al., 2014).

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Contrary to PTX2 and OA, no significant morphological changes were observed with YTX and AZA1. Nevertheless, a slight decrease of viability (around 20%) was observed with PTX2, YTX and AZA1 at the highest concentrations, reaching even up to 50% for OA. Concerning PTX2, our results on cells proportion showed that HT29-MTX cells are probably more sensitive than Caco-2 cells. This indicated that the slight decrease of viability observed for the Caco-2/HT29-MTX co-cultured cells with PTX2 might be mostly due to an effect on the HT29-MTX cell population. In fact, it has been documented that phase I enzymes play a role in PTX2 detoxification (Alarcan et al., 2017; Ferron et al., 2016b). As HT29-MTX cells do not express CYP (Carrière et al., 1994; Gervot et al., 1996; Mahler et al., 2009; Barlovatz-Meimon and Ronot, 2014), this may explain the greater sensitivity of HT29-MTX cells to PTX2 compared to Caco-2 cells. In contrast, a decrease of viability (around 20%) was reported in differentiated Caco-2 cells exposed to 200 nM PTX2 (Alarcan et al., 2019), indicating that Caco-2 cells alone seem to be more sensitive to PTX2 than in co-culture with HT29-MTX cells. Although we do not have a clear explanation for that, the mucus layer may be involved in protecting the cells. Additional studies will be necessary to verify this hypothesis. Surprisingly, YTX exposure showed a different toxicity profile with a slight decrease of the Caco-2/HT29-MTX co-cultured cells viability while no effect was observed on differentiated Caco-2 cells alone up to 200 nM YTX (Alarcan et al., 2019). This discrepancy could be related to the viability test performed: as YTX is known to disrupt lysosomes (Malagoli et al., 2006), the NRU assay (detecting lysosome activity), used in our study, is certainly more sensitive than cell counting to depict YTX toxicity. While no cytotoxicity in

451 differentiated Caco-2 cells was reported up to 72 hrs with 500 nM AZA1 (Ryan et al., 2004; Hess et
452 al., 2007; Vilarino et al., 2008; Abal et al., 2017), we observed a slight decrease of viability in
453 Caco-2/HT29-MTX co-cultured cells. Compared to the three phycotoxins mentioned above, OA
454 cytotoxicity and kinetics data on intestinal cells have been extensively published. IC₅₀ ranged from
455 200 to 400 nM after 24 hrs of exposure on differentiated Caco-2 cells (Ehlers et al., 2011; Alarcan et al.,
456 2019; Dietrich et al., 2019), suggesting a higher sensitivity of the Caco-2 cells cultured alone than in
457 co-culture with HT29-MTX cells (IC₅₀ = 550 nM). Once again, the presence of the mucus layer may
458 protect Caco-2 cells against OA. Similarly to PTX2, HT29-MTX cells seem to be more sensitive to OA
459 than Caco-2 cells (as suggested by the decrease of HT29-MTX cells percentage in the co-culture).
460 Indeed, the higher sensitivity of non-differentiated HT29-MTX cells compared to non-differentiated
461 Caco-2 cells to OA was shown by Ferron et al (2014). Again, a higher detoxification of OA by Caco-2
462 cells through P-gP and CYP activity (Ehlers et al., 2014; Kittler et al., 2014; Ferron et al., 2016b) cannot
463 be excluded. Therefore, the two cell types showed an obvious difference in sensitivity to toxins that
464 may be driven by their own detoxification capacities.

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466 For Caco-2/HT29-MTX co-cultured cells, two toxins induced a concentration-dependent alteration of
467 the two markers of monolayer integrity (a decrease of TEER values correlated with an increase of LY
468 crossing). For PTX2, concomitantly to a decrease of TEER, a decrease of *CLD1* expression and a
469 cytoplasmic redistribution of ZO-1 were observed at the highest concentration. This is consistent
470 with PTX2 targeting F-actin (Espina et al., 2008). Our results of TEER and LY crossing indicated that
471 OA up to 150 nM did not affect the Caco-2/HT29-MTX monolayer integrity, in accordance with other
472 studies performed on Caco-2 cells monolayer (Ehlers et al., 2011; Fernandez et al., 2014). For OA
473 concentrations above 150 nM, alteration of the monolayer integrity was linked to morphological
474 changes (rapid swelling and cell rounding and the formation of areas without cells (figures 2 and 5).
475 We also observed a cytoplasmic redistribution of ZO-1, a tight-junction protein commonly used for
476 investigating the effect of compounds on intestinal barrier integrity. A slight down-regulation of the
477 tight junction *CLD1* gene was also induced by OA. Similarly, a decrease of both gene expression and
478 proteins levels of tight junction proteins has been already described in differentiated Caco-2 cells
479 exposed to 150 nM OA (Dietrich et al., 2019). A cytoplasmic redistribution of ZO-1 has been also
480 reported in mouse mammary epithelia 31EG4 cells exposed to OA (Singer et al., 1994). AZA1
481 induced some alteration of permeability observed by a slight increase of LY crossing while YTX did
482 not significantly affect monolayer permeability. In contrast, moderate (Abal et al., 2017) to strong
483 (Hess et al., 2007) decrease of TEER was reported for Caco-2 cells exposed up to 100 nM AZA1.
484 Although we did not observe much cytoplasmic redistribution of ZO-1 in Caco-2/HT29-MTX
485 co-cultured cells treated with AZA1, modification of the distribution of other tight junction
486 components, such as occludin and E-cadherin, has been reported in differentiated Caco-2 cells
487 (Bellocci et al., 2010; Abal et al., 2017). Even if a decrease of *AQP3* expression has been shown to be
488 correlated with a decrease of TEER, occludin and claudin-1 expressions (Zhang et al., 2011; Ikarashi
489 et al., 2016; Ikarashi et al., 2019), in our study, YTX strongly decreased the *AQP3* expression without
490 modification of the TEER values. In contrast, the down-regulation of *AQP3* was weaker for OA,
491 although an increase of permeability was observed. Thereby, we did not find that *AQP3* expression

492 and TEER were strongly correlated, and the discrepancy with the literature data requires further
493 investigation.

494 Other parameters such as inflammation mediators can be involved in the alteration of the
495 Caco-2/HT29-MTX monolayer integrity (Sedgwick et al., 2002). Indeed, inflammation has been
496 shown to be involved in the alteration of intestinal cells monolayer integrity, particularly through
497 the release of IL-8, IL-1 β (Al-Sadi et al., 2008), TNF α (Ma et al., 2004; Al-Sadi et al., 2016) and IFN γ
498 (Beaurepaire et al., 2009). Therefore, we have evaluated if the decrease of Caco-2/HT29-MTX
499 monolayer integrity could be linked to IL-8 release, a marker of inflammation commonly used for
500 investigating phycotoxins toxicity on intestinal cell lines (Alarcan et al., 2019, Ferron et al., 2016c). In
501 this co-culture model in 96-well plates, the basal level of IL-8 release was very close to that of Caco-2
502 cells alone (Alarcan et al., 2019). We observed that OA exposure induced a large increase of IL-8
503 expression and protein release. Similarly, an increase of IL-8 release (around 30-fold) has been
504 reported in differentiated Caco-2 cells exposed to OA but at a lower concentration (38 nM) (Alarcan
505 et al., 2019). As already suggested for viability results, the mucus layer produced in co-cultures may
506 protect Caco-2 cells against OA toxicity, leading to IL-8 release with higher OA concentrations
507 compared to Caco-2 cells alone (Alarcan et al., 2019). The inflammatory response (IL-8 release) was
508 in accordance with NF- κ B translocation observed following OA treatment in intestinal epithelial and
509 glial cells (Ferron et al., 2014; Reale et al., 2019). NF- κ B is involved in inflammation as precursor of
510 cytokines release as well as in gut homeostasis (Gupta et al., 2010).

511 With PTX2, we observed a slight increase in IL-8 release only at the highest concentration tested (260
512 nM) (Figure 6). This response was not supported by an increase of IL-8 gene expression that, in
513 contrast, was slightly -but non-significantly- down-regulated. In agreement with our observations,
514 no IL-8 release was reported previously in differentiated Caco-2 cells treated with 200 nM PTX2
515 (Alarcan et al., 2019). Finally, the absence of effect on IL-8 release and gene expression after exposure
516 of Caco-2/HT29-MTX co-cultured cells to YTX is consistent with the results reported in differentiated
517 Caco-2 cells (Alarcan et al., 2019). Still, no study has investigated the effect of AZA1 on inflammation
518 in intestinal cells. However, *in vivo* studies on rodents exposed to YTX or AZA1 pointed out the
519 presence of infiltrated lymphocytes, reflecting an inflammatory area in the gut submucosa
520 (Franchini et al., 2010), the presence of neutrophils in the lamina propria (Aune et al., 2012; Kilcoyne
521 et al., 2014) as well as the infiltration of inflammatory cells in lung and liver (Ito et al., 2002).
522 Therefore, it could be suggested that the inflammatory response observed in rodents was driven by
523 another pathway than IL-8 or by another cell type than enterocytes or goblet cells.

524 Both acute stress and cytokines release, such as IL-8, can stimulate mucus secretion (Hansvall and
525 Ianowski, 2014, Hart and Kamm, 2002). Surprisingly, the impact of phycotoxins on the intestinal
526 mucus production *in vitro* has never been evaluated. In Caco-2/HT29-MTX co-cultured cells, OA
527 slightly increased the expression of *MUC13*, a transmembrane mucin, as well as *PLIN2*, a gene
528 involved in lipid droplet structure for mucins. Lipid droplets transport mucins from the intracellular
529 compartment to the apical side of goblet cells. Evaluation of mucus staining showed a slight increase
530 of acidic mucins area at 150 nM OA, although not significant. In fact, the mucus increase is expected
531 to reflect mainly the response of HT29-MTX cells to toxins. Therefore, although the percentage of

532 HT29-MTX cells was not affected until 200 nM OA, the low percentage of HT29-MTX cells in the
533 co-culture could explain why the response on mucus was not significant. In co-cultures, it is not
534 excluded that the secretion of cytokines/chemokines by Caco-2 cells stimulates HT29-MTX cells
535 resulting in an inflammatory response and perhaps mucus secretion. For PTX2, although the AB and
536 PAS stainings did not reveal any significant modification of mucus area, a slight increase of MUC12
537 was observed at the highest concentration, concomitantly with a decrease of HT29-MTX cell
538 population. In contrast, YTX and AZA1 did not affect mucins expression but a slight increase of the
539 acidic and neutral mucins area was observed at 80 nM AZA1 and 220 nM YTX respectively.
540 Therefore, it could be worth measuring mucus production of HT29-MTX cells alone in response to
541 phycotoxins exposure. Moreover, post-transcriptional O-glycosylation and N-glycosylation of the
542 mucins could be also a pathway to investigate as the aberrant glycan structure of mucins induces
543 perturbation of the gut functions and gut homeostasis (Bergstrom and Xia, 2013; Hanson and
544 Hollingsworth, 2016; Arike et al., 2017).

545 5. Conclusions

546 In conclusion, our study investigated the mode of action of 4 phycotoxins using a co-culture
547 intestinal model composed of enterocytes-like (Caco-2) and mucus producing cells (HT29-MTX). We
548 explored how the responses of such model fit with the observations in humans and rodents. Our
549 results indicated that the mucus layer could protect Caco-2 cells from OA toxicity. The HT29-MTX
550 cell population appeared more sensitive to OA and PTX2 than Caco-2 cells. In contrast, YTX and
551 AZA1 induced a slight cytotoxicity, expressed mainly by a slight decrease of monolayer integrity.
552 Our study highlighted that intestinal cell models integrating different cell types may contribute to
553 improve hazard assessment and to describe how phycotoxins can affect human intestinal tract.
554 Therefore, the Caco-2/HT29-MTX is a suitable cell model combining the two major epithelial cells for
555 the evaluation of phycotoxins toxicity on the intestinal barrier. Nevertheless, it does not contain cell
556 types from immune and nervous systems that are also known as key players in the toxic response of
557 food contaminants. Although more complex cell models are developed to integrate such cell types,
558 they are still not validated to replace *in vivo* studies.

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560

561 **Author Contributions:** Conceived and designed the experiments: OR, AH, VF. Performed the experiments: OR.
562 Analyzed the data: OR, AH, VF. Wrote the paper: OR, AH, VF.

563 **Acknowledgements:** We thank Gérard Jarry (ANSES Laboratory Fougères) and Alain Fautrel (University
564 Rennes 1, US18, UMS 3480 Biosit, Biogenouest, Core Facility H2P2) for their support in histology.

565 **Conflicts of Interest:** The authors declare no conflict of interest

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