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Combined effects of okadaic acid and pectenotoxin-2, 13-desmethylspirolide C or yessotoxin in human intestinal Caco-2 cells

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Abstract (150-250 words)

Lipophilic phycotoxins are secondary metabolites produced by phytoplanktonic species. They accumulate in filtering shellfish and can cause human intoxications. Humans can be exposed to combinations of several phycotoxins. The toxicological effects of phycotoxin mixtures on human health are largely unknown. Published data on phycotoxin co-exposure show that okadaic acid (OA) is simultaneously found with pectenetoxin-2 (PTX-2), 13-desmethylspirolide C (also known as SPX-1), or yessotoxin (YTX). Therefore, the aim of this study was to examine the effects of three binary mixtures, OA/PTX-2, OA/SPX-1 and OA/YTX on human intestinal Caco-2 cells. A multi-parametric approach for cytotoxicity determination was applied using a high-content analysis platform, including markers for cell viability, oxidative stress, inflammation, and DNA damage. Mixtures effects were analyzed using two additivity mathematical models. Our assays revealed that OA induced cytotoxicity, DNA strand breaks and interleukin 8 release. PTX-2 slightly induced DNA strand breaks, whereas SPX-1 and YTX did not affect the investigated endpoints. The combination of OA with another toxin resulted in reduced toxicity at low concentrations, suggesting antagonistic effects, but in increased effects at higher concentrations, suggesting additive or synergistic effects. Taken together, our results demonstrated that the cytotoxic effects of binary mixtures of lipophilic phycotoxins could not be predicted by additivity mathematical models. In conclusion, the present data suggest that combined effects of phycotoxins may occur which might have the potential to impact on risk assessment of these compounds.

Keywords: phycotoxins, mixtures, antagonism, synergism, genotoxicity, inflammation

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Abbreviations[1]: ABC: ATP-binding cassette transporter; ADME: absorption, distribution, metabolism, excretion; CI: combination index; CYP: cytochrome P450; DSP: diarrhetic shellfish poisoning; EFSA: European Food Safety Authority; IL-8: interleukin 8; OA: okadaic acid; P-gp: P-glycoprotein; PP2A: protein phosphatase 2A; PTX-2: pectenotoxin-2; PXR: pregnane-X-receptor; ROS: reactive oxygen species; SM: shellfish meat; SPX-1: 13-desmethylspirolide C; TEF: toxicity equivalent factor; YTX: yessotoxin.

1. Introduction (750 words)

Marine biotoxins are secondary metabolites produced by specific phytoplanktonic species (Visciano et al., 2016). Among the lipophilic marine biotoxins, four main families have been described: okadaic acid (OA) and dinophysistoxins (DTXs), pectenotoxins (PTXs), yessotoxins (YTXs) and finally cyclic imines (including spirolides, pinnatoxins, pteriatoxins and gymnodimines). OA and DTXs are responsible for diarrhetic shellfish poisoning (DSP), characterized by diarrhea, nausea, abdominal pain and vomiting (Valdiglesias et al., 2013). The OA group of toxins inhibits serine/threonine protein phosphatase 2A (PP2A) and, to a lesser extent, also PP1 (Takai et al., 1992). The group of PTXs, principally PTX-2, is not anymore considered a DSP toxin since its implication in gastro-intestinal symptoms is not clear (Ito et al., 2008). At the cellular level, PTX-2 provokes actin depolarization leading to a disruption of the cytoskeleton (Alligham et al., 2007). The group of YTXs has no recorded effects in humans but has been frequently found together with OA in shellfish (Alarcan et al., 2018). YTX displays *in vitro* toxicity, for instance apoptosis induction and disturbance of the calcium flux (Tubaro et al., 2008). Its mechanism of action is still unknown but some studies suggest a possible connection with autophagy (Fernández-Araujo et al., 2015). Finally, potent neurological effects in mice have been reported for the group of cyclic imines (Munday et al., 2012). SPX-1 is a selective inhibitor of nicotinic acetylcholine receptors (Araoz et al., 2015).

For some of them, regulatory limits were set to protect consumers from their potential harm as they can contaminate shellfish and be responsible for foodborne outbreaks (EFSA report 2009). OA and PTX-2 together share a limit of 160 µg /kg shellfish meat (SM), and for YTX and its analogues the limit is set to 3.75 mg YTX eq/kg SM. However, in the absence of reports on human intoxications, no regulatory limits have been established for the group of cyclic imines, even though they are frequently detected in shellfish and can be very toxic to mice following intra-periteonal or oral administration (Munday et al., 2012). Mixtures of marine biotoxins are only regarded by the abovementioned EFSA opinion in case of toxin analogs with established toxicity equivalent factors (TEFs). However, studies regarding deleterious effects generated by the mixtures of toxins from different chemical groups are lacking. Therefore the impact of toxin co-occurrence on toxicity needs to be more deeply investigated.

A high number of different approaches have been described to analyze the combined effects of mixtures (Foucquier and Guedj 2015). Even though antagonism and synergism are well-established

[1]**Abbreviations:** ABC: ATP-binding cassette transporter; ADME: absorption, distribution, metabolism, excretion; CI: combination index; CYP: cytochrome P450; DSP: diarrhetic shellfish poisoning; EFSA: European Food Safety Authority; IL-8: interleukin 8; OA: okadaic acid; P-gp: P-glycoprotein; PP2A: protein phosphatase 2A; PTX-2: pectenotoxin-2; PXR: pregnane-X-receptor; ROS: reactive oxygen species; SPX-1: 13-desmethylspirolide C; TEF: toxicity equivalent factor; YTX: yessotoxin.

concepts, the definition of additivity is broad (Foucquier and Guedj, 2015) resulting in several proposed models. Generally speaking, additivity models allow predicting the additive effects of a mixture from the toxicity of each component. They assume that each component exerts an effect without influencing the action of the other ones. Deviation from predicted additivity is considered as synergism or antagonism. The two main concepts used are concentration addition (CA) and independent action (IA). The CA concept relies on the assumption that the components in the mixture act on the same biological sites, by the same mechanisms of action and differ only in their potency (Loewe and Muischnek, 1926). The IA concept established by Bliss (1939) relies on the hypothesis that a combined effect can be calculated from the responses of the individual mixture components, assuming that each one acts independently without interfering with the others (different sites of action).

So far, only few studies have been undertaken with mixtures of phycotoxins. No combined effects were reported in rodents except in the distribution into internal organs where a reduced level was observed in case of mixtures (Aasen et al., 2011; Sosa et al., 2013). In human neuroblastoma cells, OA co-incubated with YTX or SPX-1 showed additive effect towards cell viability (Rodriguez et al., 2015). However, on proliferative human intestinal epithelial cells, binary combinations of azaspiracid-1 (AZA-1)/OA and YTX/OA resulted in an increasing antagonistic effect on viability when toxin concentrations raised, whereas AZA-1/YTX mixtures showed dose-dependent synergism (Ferron et al., 2016).

In this study we chose relevant mixtures based on our recently published review (Alarcan et al., 2018), highlighting that OA was predominantly found as the main toxin combined with PTX-2, YTX or SPX-1. Our objective was to complete the previous data by assessing a panel of different toxicity endpoints (cell viability, oxidative stress, inflammation, and DNA strand breaks) in the human Caco-2 cell line with binary mixtures of lipophilic toxins.

2. Materials and methods

2.1 Establishment of mixtures

Three binary mixtures were selected in the following molar ratios according to published literature on lipophilic phycotoxins co-exposure (Alarcan et al., 2018): 3:1 for OA/PTX-2 and OA/YTX and 9:1 for OA/SPX-1. Concentrations ranges were established based on preliminary cell viability experiments and published data. PTX-2, YTX and SPX-1 did not show any cytotoxicity (NRU, DAPI cell count) after 24h on differentiated Caco-2 cells up to 500, 200 and 510 nM, respectively (unpublished data). Based on the cytotoxicity results (MTT assay) for OA reported by Ehlers et al. (2011), a maximum concentration of 600 nM was chosen in order to study biological responses in both cytotoxic and non-cytotoxic conditions. Then, based on the ratios of toxins previously indicated, we deduced the concentrations for PTX-2, YTX and SPX-1 from those chosen for OA.

2.2 Chemicals

Toxin standards were purchased from the National Research Council Institute for Marine Biosciences (Halifax, NS Canada). DCFH-DA, potassium bromate ($KBrO_3$), sulfuric acid (H_2SO_4) and TNFα were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary anti-interleukin 8 (IL-8) antibody (M801), biotin-conjugated human IL-8 (M802B), HRP-conjugated streptavidin (N100), and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Mouse monoclonal anti γH2AX S19 (ab26350) and goat pAb to Ms IgG Alexa Fluor 647 (ab150115)

were purchased from Abcam (Cambridge, UK). All other chemicals including ethanol (EtOH), methanol (MeOH) and dimethyl sulfoxide (DMSO) were of analytical grade and purchased from Fisher Scientific (Leicestershire, England). Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.3 Cell culture

Caco-2 cells were obtained from the American Type Culture Collection HTB-37 (LGC Standards, Molsheim, France). Cells (passages 30–38) were seeded at 10,000 cells/cm^2 in 96-well plates in culture medium (minimum essential medium, supplemented with 10% fetal calf serum, 1% non-essential amino acids, 50 IU/ml penicillin and 50 µg/ml streptomycin) and maintained at 37 °C under 5% CO_2. Media, serum, amino acids solution and antibiotics were all from Invitrogen (Saint Aubin, France). For differentiation into an intestinal epithelial-like monolayer, cells were cultured for 3 weeks with renewal of medium every 2 to 3 days (Sambuy et al., 2005). All the assays were performed on differentiated Caco-2 cells.

2.4 Measurement of ROS production

ROS were measured using the probe DCFH-DA. Caco-2 cells were pre-treated with 25 µM DCFH-DA for 30 min. The culture medium was then removed and medium containing the toxins was added for 24 h of incubation at 37°C. Fluorescence was measured at λ ext = 485 nm and λ em = 520 nm using a Fluostar Omega microplate reader (BMG Labtek).

2.5 Cell viability and DNA strand breaks

After 24 h of treatment with the toxins, Caco-2 cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% Triton X-100 for 15 min. Plates were then incubated in blocking solution (PBS with 1% BSA and 0.05% Tween-20) for 30 min before the addition of primary antibody prepared in blocking solution and filtered with a 0.2 µm syringe filter. Mouse monoclonal anti γH2AX S19 (1:1000 dilution) was incubated with the cells for 1.5 h at room temperature. After washing with PBS + 0.05% Tween-20, goat pAb to Ms IgG Alexa Fluor 647 (1:1000) was added for 45 min incubation at room temperature. Nuclear DAPI (1 µg/mL) staining was used for automated cell identification by high content analysis. Plates were scanned with the Thermo Scientific ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, USA) and analyzed using the Target Activation module of the BioApplication software. For each well, 10 fields (10X magnification) were scanned and analyzed for immunofluorescence quantification. Cell counts were determined by DAPI staining. γ-H2AX was quantified in the nuclei and expressed as fold increase compared to solvent controls. No γ-H2AX data are provided for toxin concentrations resulting in a cell viability < 40%, to ensure a reliable measurement of DNA damage.

2.6 IL-8 release by ELISA

After treatment with toxins, cell culture supernatants were collected and analyzed for IL-8 release. Samples were transferred to 96-well microplates coated overnight at 4°C with 1 µg/mL human recombinant anti-IL-8 primary antibody. Following addition of 0.1 µg/mL of the biotin-conjugated human IL-8 antibody for 1 h, streptavidin peroxidase (1:10000) was added for 45 min. After washing with PBS + 0.05% Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to initiate the colorimetric reaction. The reaction was stopped by 1 M H_2SO_4 and absorbance was measured at 405 nm using a Fluostar Omega microplate reader (BMG Labtek).

2.7 Statistics/Data analysis

GraphPad Prism 5 (GraphPad Software, Inc) was used for statistical analyses. Two-way ANOVA followed by Bonferroni's post-hoc tests were used to compare the effects of toxins and solvent control All error bars denote standard error of the mean (SEM). Symbols *, **, ***, **** and #, ##, ###, #### indicate statistical significance between toxin and solvent control or OA and mixture respectively ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ respectively).

2.8 Mixture effects predictions using additivity models

Since the tested phycotoxins do not share any known common mechanism of action, using the CA concept is therefore not relevant. Instead, the mathematical definition of additivity, also known as theoretical additivity, response additivity or linear interaction effect, was employed (Foucquier and Guedj, 2015). The independent action concept was also employed.

2.8.1 Theoretical additivity method

The theoretical additivity (TA) method was used as described by Weber et al., (2005). This method compares theoretical predicted values calculated from the results of each individual compound to the measured values obtained with mixtures. In our study of binary phycotoxin combinations, the predicted mixture effect values were calculated as follows:

$$Mix(A+B)predicted\ value = (mean\ value\ A + mean\ value\ B) - mean\ value\ solvent\ control$$

For data expressed as fold change compared to solvent control, mean value solvent control = 1 while for cell viability analysis, the mean value for the solvent control was set to 100.
Combination index (CI), a standard measure of combination effect, was calculated as indicated by Foucquier and Guedj (2015):

$$CI = \frac{Mix(A+B)predicted\ value}{Mix(A+B)measured\ value}$$

Threshold of the computed CI of CI< 0.9, $0.9 \leq CI \leq 1.1$, and CI > 1.1 were set to indicate synergism, additive effects and antagonism, respectively. In order to get cell viability CI values that respect the combination effect range, ratios $\frac{1}{CI}$ were calculated.

2.8.2 Independent action concept

The independent action (IA) concept was used as described by Foucquier and Guedj (2015). This method established by Bliss (1939) relies on the hypothesis that a combined effect can be calculated from the responses of the individual mixture components, assuming that each one acts independently without interfering with the others (different sites of action). In our study, for binary phycotoxin combinations, the predicted mixture effect values were calculated as follows:

$$Mix(A+B)predicted\ value = (mean\ value\ A + mean\ value\ B) - (mean\ value\ A \times mean\ value\ B)$$

The CI were calculated as indicated by Foucquier and Guedj (2015):

$$CI = \frac{Mix(A+B)predicted\ value}{Mix(A+B)measured\ value}$$

To establish the predictions, the raw data of ROS production, IL-8 release and γ-H2AX phosphorylation, originally expressed as fold change compared to the respective solvent control, were processed to obtain data suitable for IA analysis, i.e. comprised between 0 and 1. For each endpoint, the condition among the phycotoxins tested individually and the binary mixture showing the highest effect was attributed the value of 1 (Amax). All other conditions were then normalized to the Amax. This data processing was described previously by Alassane-Kpembi et al. (2016) and allows assessing combined effects for endpoints other than cell viability. The raw data of cell viability were first divided by 100 to get data comprised between 0 and 1. Cell viability values superior to 100 were set to 1. Cell viability CI values were calculated as described for TA.

3. Results

3.1 Toxic effects following OA/PTX-2 mixtures treatment in Caco-2 cells

3.1.1 PTX-2 exacerbates the cytotoxicity of OA in Caco-2 cells

PTX-2 alone was not toxic for Caco-2 cells, whereas OA showed concentration-dependent toxicity (IC_{50} = 331 nM) (Fig. 1a). The binary OA/PTX-2 mixture induced strong cytotoxicity in Caco-2 cells, with a tremendous decrease of cells number (80% for the condition D) (Fig. 1a), indicating that the addition of PTX-2 increased the cytotoxicity of OA.

3.1.2 PTX-2 decreases ROS production induced by OA

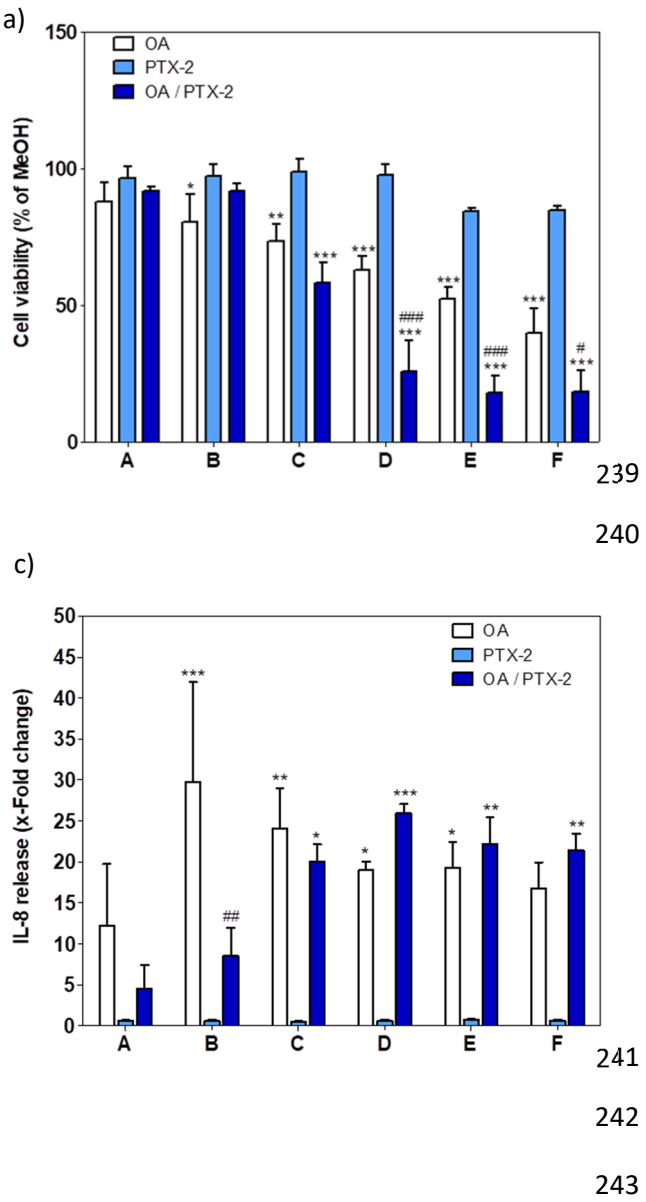
After a 24 h treatment, OA not-statistically increased ROS production while PTX-2 had no effect (Fig. 1b). We observed that low and intermediate concentrations of OA/PTX-2 (conditions B and C) reduced the level of ROS production compared to that induced by OA alone (Fig. 1b).

3.1.3 PTX-2 decreases IL-8 release induced by OA only at low concentrations

Only OA induced IL-8 release in Caco-2 cells (conditions B to E) (Fig. 1c). For low concentrations the OA/PTX-2 mixture resulted in a reduction of IL-8 release compared to that induced by OA alone, which was statistically significant for 37.5/12.5 nM (condition B). PTX-2 did not decrease IL-8 production induced by OA at higher concentrations (Fig. 1c).

3.1.3 PTX-2 decreases γ-H2AX phosphorylation induced by OA at low concentrations

PTX-2 alone slightly induced the phosphorylation of γ-H2AX (1.8-fold, not statistically significant) whereas OA concentration-dependently induced the phosphorylation of γ-H2AX even at low concentration (37.5 nM condition B, or higher) (Fig. 1d). For 37.5/12.5 nM (condition B), the mixture resulted in a lower phosphorylation of γ-H2AX compared to that induced by OA alone (Fig. 1d). This mixture effect is also illustrated by the immunostaining in Fig. 2. No data are provided for the three highest concentrations due to the high cytotoxicity that prevents from a reliable measurement of DNA damage.



nM	solo		mixtures
	OA	**PTX-2**	**OA/PTX-2**
A	18.75	6.25	18.75/6.25
B	37.5	12.5	37.5/12.5
C	75	25	75/25
D	150	50	150/50
E	300	100	300/100
F	600	200	600/200

Fig. 1 Effects of OA/PTX-2 mixtures on a panel of toxicity endpoints in differentiated Caco-2 cells. Cells were pre-treated with the ROS probe DCFH-DA (25 µM) for 30 min before incubation with the toxin(s) for 24 h. After fluorometric measurement of ROS production, cell culture supernatant was collected for the quantification of IL-8 release, while cells were fixed for DAPI and γ-H2AX immunostaining: (a) cell viability, (b) ROS production, (c) IL-8 release and (d) phosphorylation of γ-H2AX are shown. $KBrO_3$ (500 µM) and TNFα (20 ng/ml) were used as positive controls. $KBrO_3$ increased ROS production by 1.5-fold and induced the phosphorylation of γ-H2AX by 2.7-fold. TNFα failed to induce IL-8 release. Results were obtained from three independent experiments. Data represents means ± SEM of fold change compared to solvent control. *, **, *** and #, ##, ### indicate statistical significance between toxin and solvent control or OA and mixture respectively ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively) after two-way ANOVA followed by Bonferroni's post-hoc test. §: no data are provided due to high cytotoxicity that prevents from a reliable measurement of DNA damage.

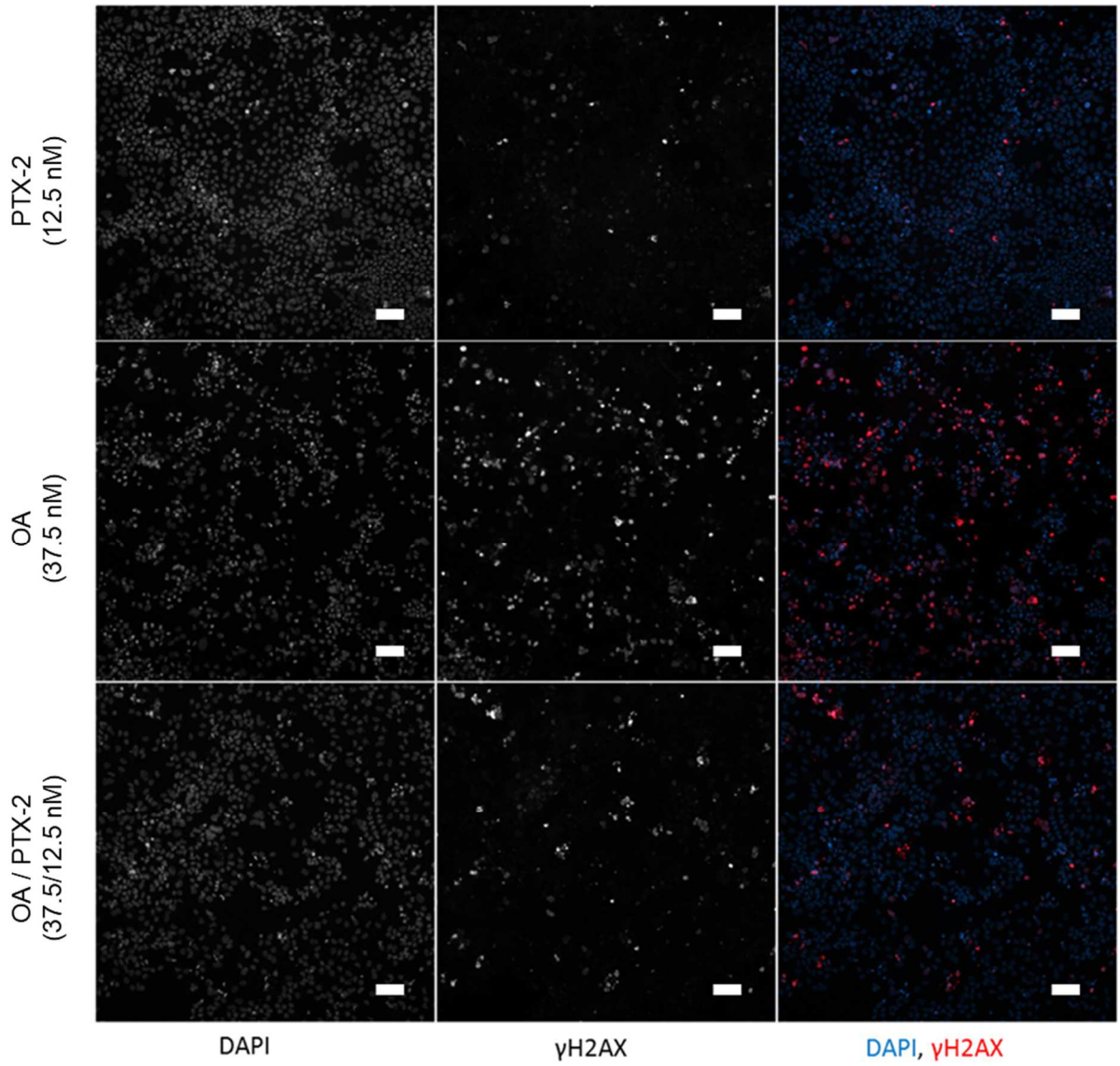


Fig. 2 Representative images (10X magnification) of DNA damage induced by OA/PTX-2 mixtures. Cells were incubated with toxins for 24 h and labeled with antibodies against γ-H2AX. The nuclei were stained with DAPI and images were captured with an Arrayscan VTi. White bar = 100 µm.

3.2 Toxic effects following OA/SPX-1 mixtures treatment in Caco-2 cells

3.2.1 SPX-1 reduces the cytotoxicity effect of OA in Caco-2 cells at low concentrations

SPX-1 was not toxic for Caco-2 cells in the tested range concentration. In mixture condition, a lower toxicity was depicted for low and intermediate concentrations (conditions B and C) even if not statistically significant (Fig. 3a).

3.2.2 SPX-1 decreases ROS production induced by OA

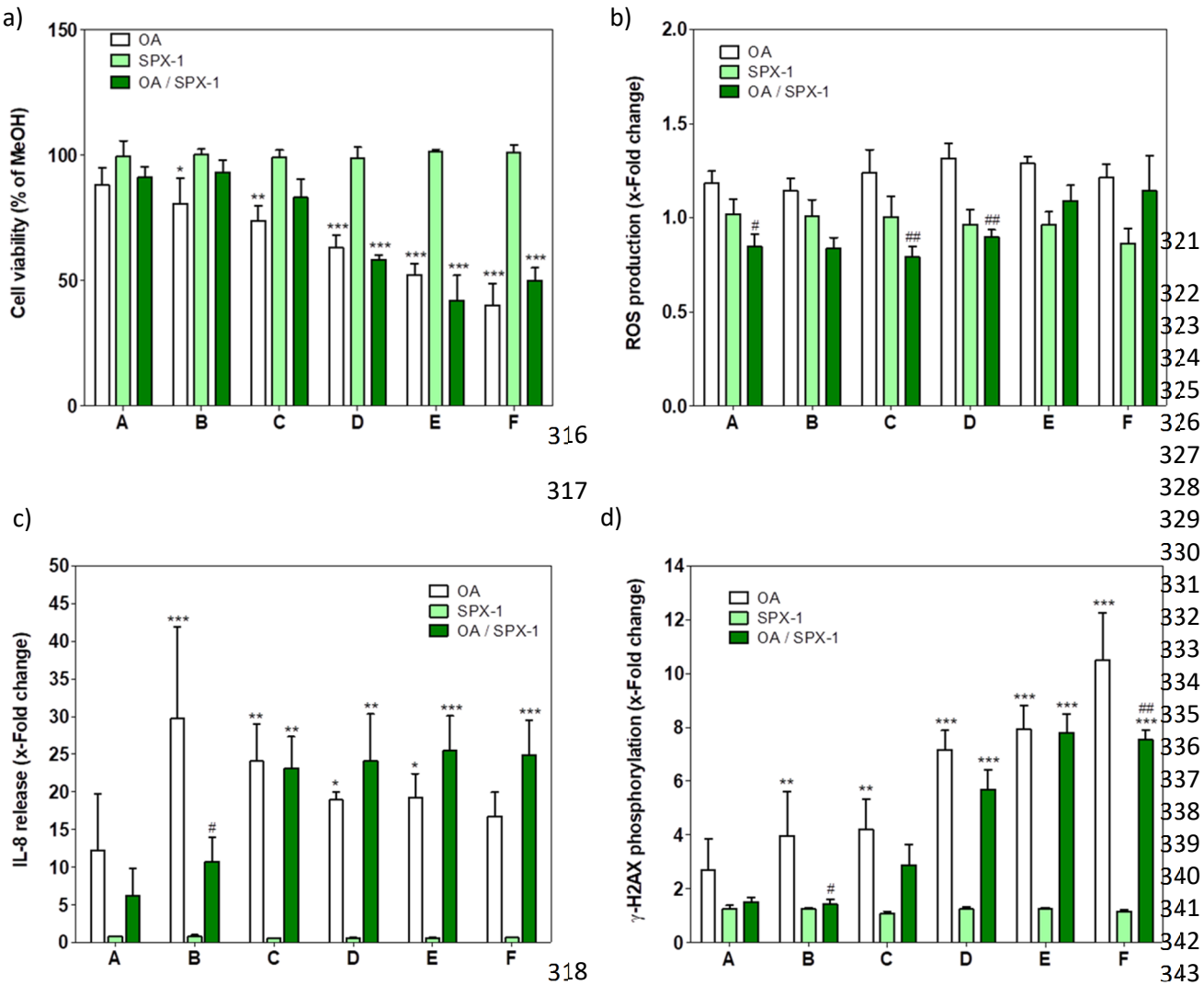
Whereas SPX-1 failed to induce ROS production, we observed that low and intermediate concentrations of OA/SPX-1 (conditions A, C and D) reduced the level of ROS production compared to that induced by OA alone (Fig. 3b).

3.2.3 SPX-1 decreases IL-8 release induced by OA only at low concentrations

SPX-1 did not induce the release of IL-8. For low concentrations the OA/SPX-1 mixture resulted in a reduction of IL-8 release compared to that induced by OA alone, which was statistically significant for 37.5/12.5 nM (condition B). SPX-1 did not decrease IL-8 production induced by OA at higher concentrations (Fig. 3c).

3.2.4 SPX-1 decreases γ-H2AX phosphorylation induced by OA at low concentrations

No induction of γ-H2AX phosphorylation was observed after treatment with SPX-1. For 37.5/12.5 and 600/200 nM (conditions B and F), the OA/SPX-1 mixture resulted in a reduced phosphorylation of γ-H2AX compared to that induced by OA alone (Fig. 1d). This mixture effect is also illustrated by the immunostaining in Fig. 4.



nM	solo		mixtures
	OA	**SPX-1**	**OA/SPX-1**
A	18.75	2.1	18.75/2.1
B	37.5	4.2	37.5/4.2
C	75	8.3	75/8.3
D	150	16.7	150/16.7
E	300	33.4	300/33.4
F	600	66.7	600/66.7

Fig. 3 Effects of OA/SPX-1 mixtures on a panel of toxicity endpoints in differentiated Caco-2 cells. Cells were pre-treated with the ROS probe DCFH-DA (25 µM) for 30 min before incubation with the toxin(s) for 24 h. After fluorometric measurement of ROS production, cell culture supernatant was collected for the quantification of IL-8 release, while cells were fixed for DAPI and γ-H2AX immunostaining: (a) cell viability, (b) ROS production, (c) IL-8 release and (d) phosphorylation of γ-H2AX are shown. $KBrO_3$ (500 µM) and TnFα (20 ng/ml) were used as positive controls. KBrO3 increased ROS production by 1.5-fold and induced the phosphorylation of γ-H2AX by 2.7-fold. TNFα failed to induce IL-8 release. Results were obtained from three independent experiments. Data represents means ± SEM of fold change compared to solvent control. *, **, *** and #, ## indicate statistical significance between toxin and solvent control or OA and mixture respectively ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively) after two-way ANOVA followed by Bonferroni's post-hoc test.

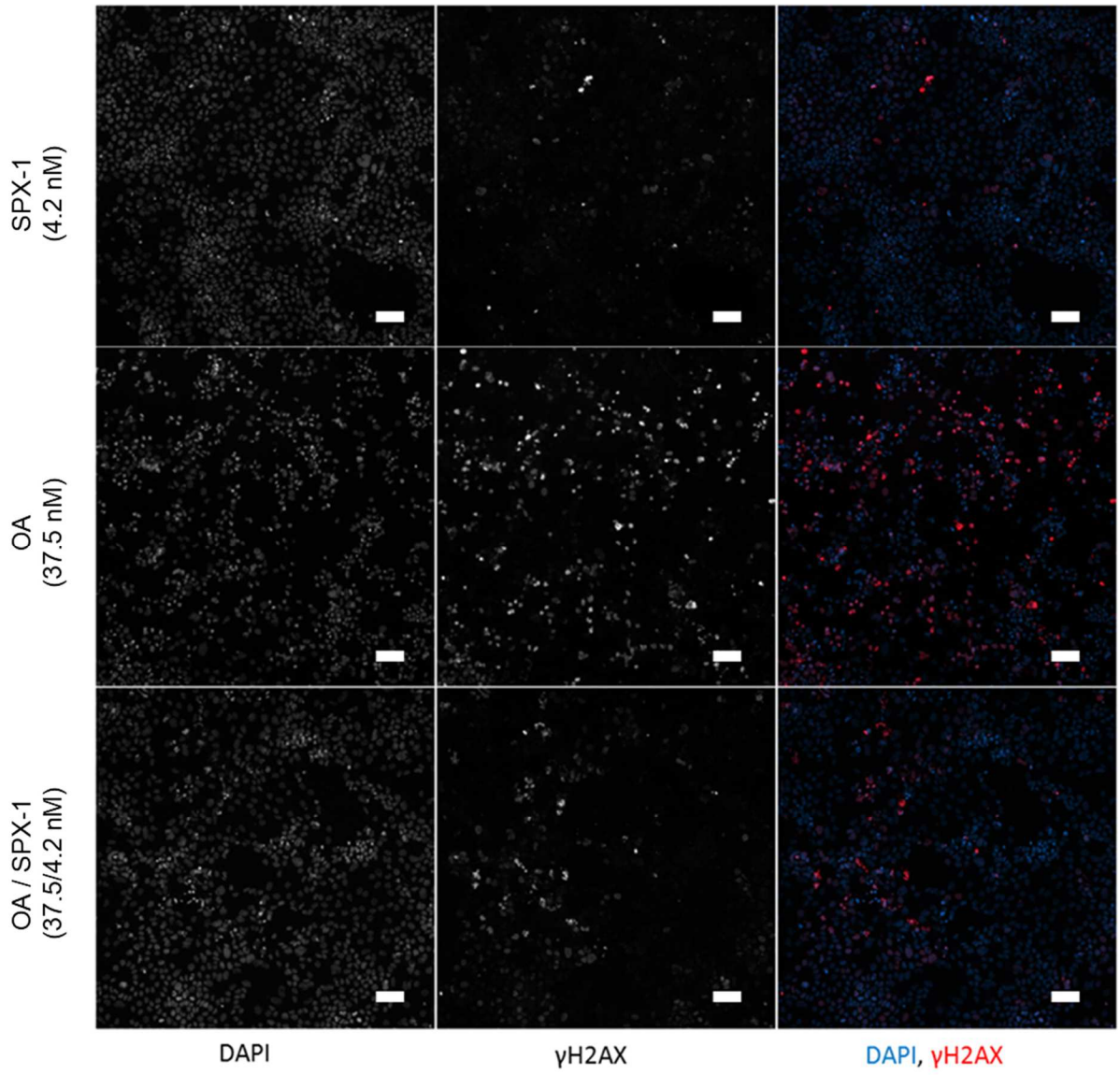


Fig. 4 Representative images (10X magnification) of DNA damage induced by OA/SPX-1 mixtures. Cells were incubated with toxins for 24 h and labeled with antibodies against γ-H2AX. The nuclei were stained with DAPI and images were captured with an Arrayscan VTi. White bar = 100 µm.

3.3 Toxic effects following OA/YTX mixtures treatment in Caco-2 cells

3.3.1 YTX reduces the cytotoxicity effect of OA in Caco-2 cells at low concentrations

YTX was not toxic for Caco-2 cells in the tested range concentration. In mixture condition, a lower toxicity was depicted for low and intermediate concentrations (conditions A and B) even if not statistically significant (Fig. 5a).

3.3.2 YTX decreases ROS production induced by OA

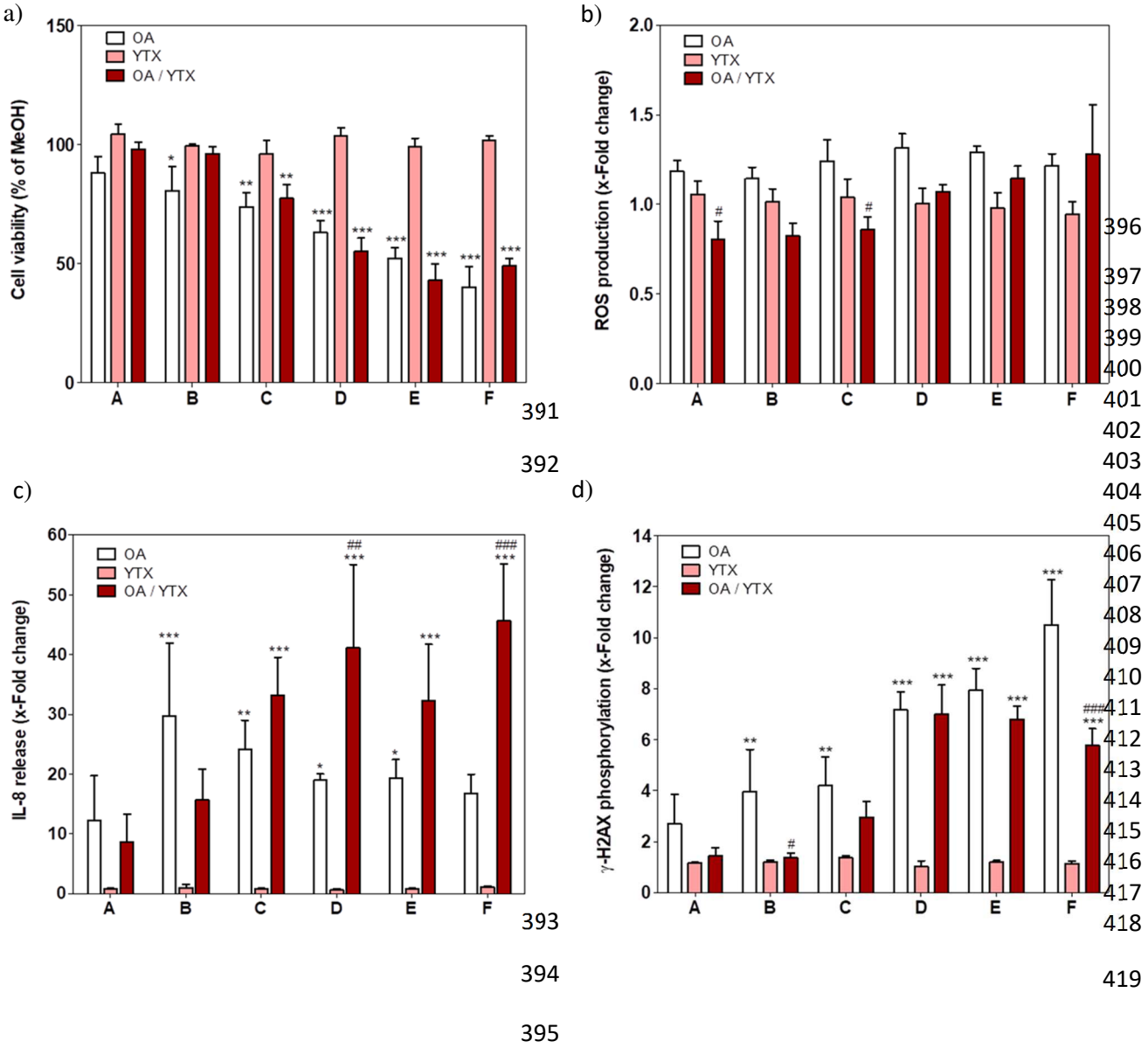
Whereas YTX failed to induce ROS production, we observed that low and intermediate concentrations of OA/YTX (conditions A and C) reduced the level of ROS production compared to that induced by OA alone.

3.3.3 YTX increases IL-8 release induced by OA at high concentrations

If YTX failed to induce IL-8 release in Caco-2 cells, the mixtures with a high concentration of YTX showed an increase of IL-8 release compared to that induced by OA alone (conditions D and F) (Fig. 5c).

3.3.4 YTX decreases γ-H2AX phosphorylation induced by OA at low concentrations

No induction of γ-H2AX phosphorylation was observed after treatment with YTX. Similarly to what was observed for the OA/SPX-1 mixture, for 37.5/12.5 and 600/200 nM (conditions B and F), the OA/YTX mixture resulted in a reduced phosphorylation of γ-H2AX compared to that induced by OA alone (Fig. 1d). This mixture effect is also illustrated by the immunostaining in Fig. 6.



nM	solo		mixtures
	OA	**YTX**	**OA/YTX**
A	18.75	6.25	18.75/6.25
B	37.5	12.5	37.5/12.5
C	75	25	75/25
D	150	50	150/50
E	300	100	300/100
F	600	200	600/200

Fig. 5 Effects of OA/YTX mixtures on a panel of toxicity endpoints in differentiated Caco-2 cells. Cells were pre-treated with the ROS probe DCFH-DA (25 µM) for 30 min before incubation with the toxin(s) for 24 h. After fluorometric measurement of ROS production, cell culture supernatant was collected for the quantification of IL-8 release, while cells were fixed for DAPI and γ-H2AX immunostaining: (a) cell viability, (b) ROS production, (c) IL-8 release and (d) phosphorylation of γ-H2AX are shown. $KBrO_3$ (500 µM) and TnFα (20 ng/ml) were used as positive controls. KBrO3 increased ROS production by 1.5-fold and induced the phosphorylation of γ-H2AX by 2.7-fold. TNFα failed to induce IL-8 release. Results were obtained from three independent experiments. Data represents means ± SEM of fold change compared to solvent control. *, **, *** and #, ##, ### indicate statistical significance between toxin and solvent control or OA and mixture respectively ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively) after two-way ANOVA followed by Bonferroni's post-hoc test.

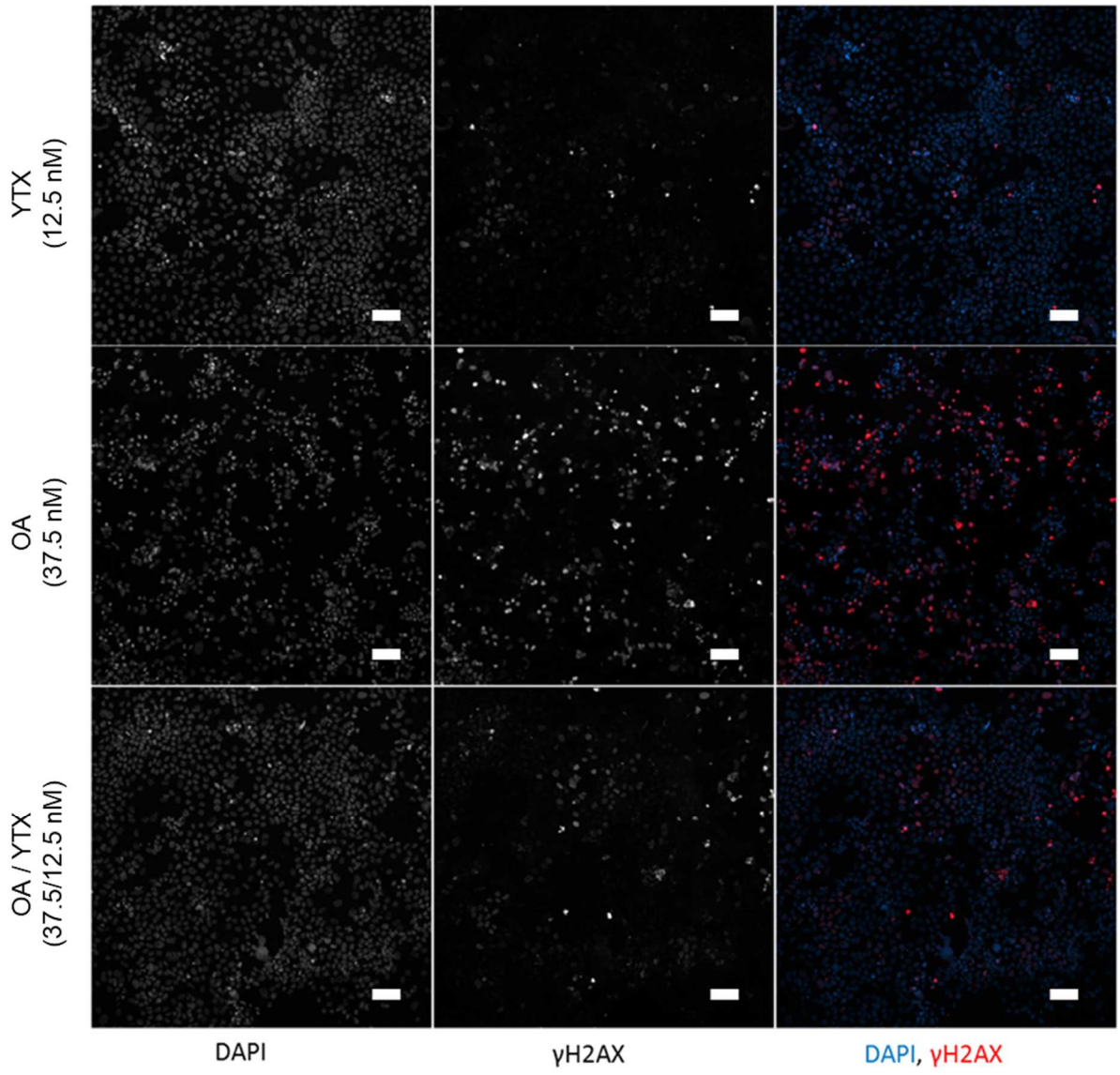


Fig. 6 Representative images (10X magnification) of DNA damage induced by OA/YTX mixtures. Cells were incubated with toxins for 24 h and labeled with antibodies against γ-H2AX. The nuclei were stained with DAPI and images were captured with an Arrayscan VTi. White bar = 100 μm.

3.4 Predictions of mixture effects using the theoretical additivity (TA) method and the independent action (IA) concept

3.4.1 OA/PTX-2 mixtures treatment on Caco-2 cells

Regarding cytotoxicity, TA model predicted additivity or antagonism for the two low concentrations while synergism occurred at higher concentrations except for the highest concentration where additivity was shown (Fig. 7a). IA model predicted additivity for the two low concentrations and synergism at higher concentrations. Using IA model, antagonism was observed for all concentrations towards ROS production while TA predicted additivity for the two highest concentrations. For γ-H2AX phosphorylation, no predictions at the three highest concentrations were made due to the high cytotoxicity that prevents from a reliable measurement of DNA damage. Both models gave similar predictions of antagonism for A and B conditions while C condition showed additivity. Antagonism towards IL-8 release was obtained for low concentrations, compared to slight synergism or additivity at higher concentrations with both models.

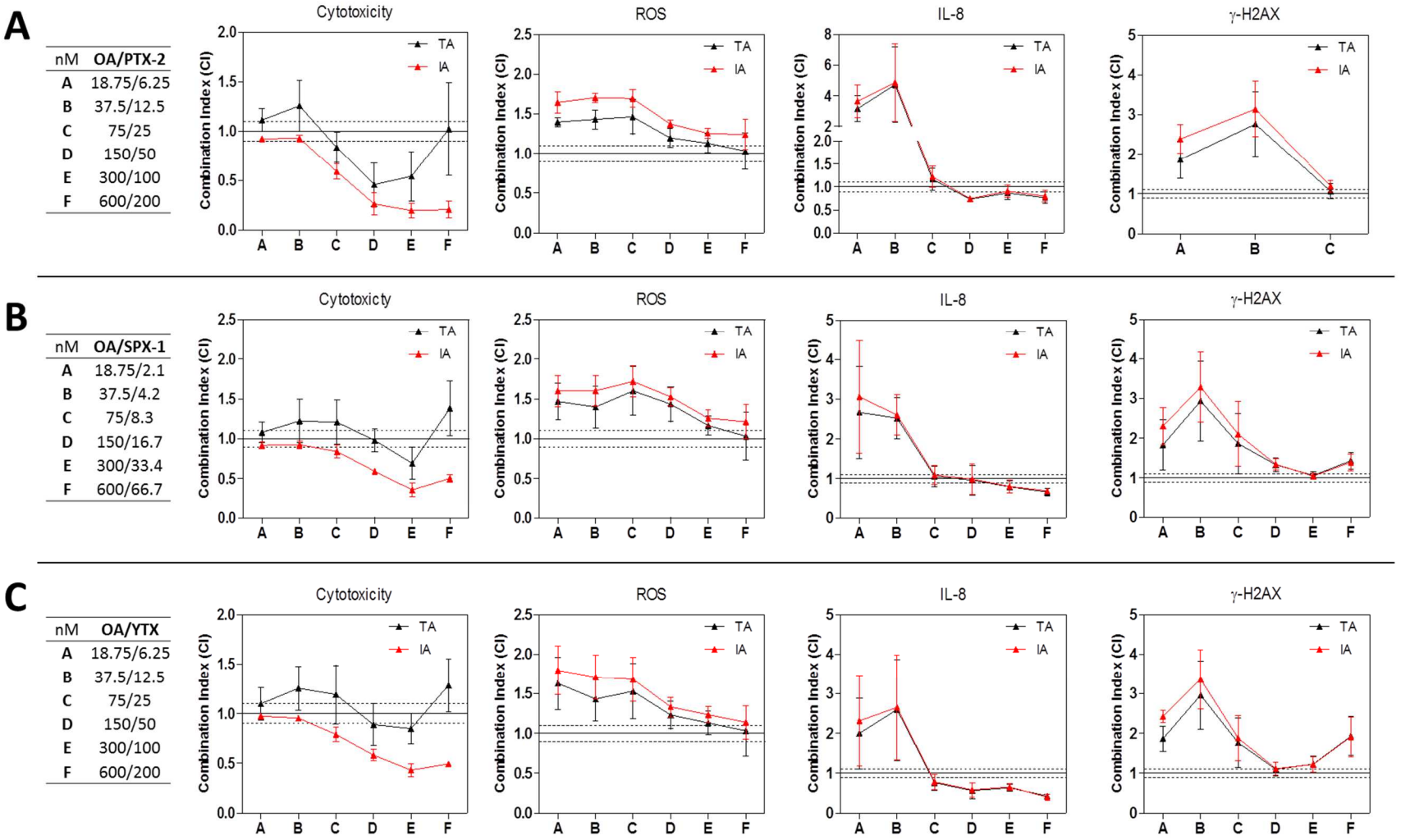
3.4.2 OA/SPX-1 mixtures treatment on Caco-2 cells

Regarding cytotoxicity, no clear tendency emerges but additivity seems to be the main effect occurring using TA model (Fig. 7b). IA model predicted additivity for the two low concentrations and synergism at higher concentrations. For IL-8 release, all kinds of effects were observed from antagonism at the lowest concentrations, to additivity or synergism at the intermediate and highest concentrations respectively. Using IA model, antagonism was observed for all concentrations towards ROS production while TA predicted additivity for the highest concentration. For γ-H2AX phosphorylation, both models predicted antagonism except for E condition where additivity was described.

3.4.3 OA/YTX mixtures treatment on Caco-2 cells

Regarding cytotoxicity, no clear tendency emerges but additivity seems to be the main effect occurring using TA model (Fig. 7c). IA model predicted additivity for the two low concentrations and synergism at higher concentrations. ROS measurement and γ-H2AX phosphorylation showed antagonism for almost all concentrations, additivity being sometimes described (e.g D condition for γ-H2AX phosphorylation or F condition for ROS with TA). Antagonism towards IL-8 release was obtained for low concentrations, compared to synergism at higher concentrations.

Fig. 7 Analysis of mixtures using the theoretical additivity (TA) method and the independent action (IA) concept on a panel of toxicity endpoints in differentiated Caco-2 cells. CI < 0.9, 0.9 ≤ CI ≤ 1.1 and CI > 1.1 indicate respectively synergism, additive effect and antagonism. Data represents means ± SEM from three independent experiments. Dashed lines indicate lower and upper limits of additivity.



4. Discussion (1500 words)

In this study, we investigated the effects of binary mixtures of lipophilic phycotoxins on a variety of toxicity endpoints in a human intestinal cell model. The mixture compositions and ratios were selected according to a review of the literature on phycotoxin mixtures (Alarcan et al., 2018). Three main mixtures were tested, all of them featuring OA as the major constituent and reflecting realistic exposure scenarios. Irrespective of the phycotoxin combined with OA, the effects observed were, according to the chosen mathematical approach, mainly antagonistic at low concentrations and, in a few cases, synergistic at higher concentrations.

When investigating mixture effects, the choice of endpoints is a main concern. A specific endpoint can be easily selected when the compounds share a common mechanism of action. In our case, the toxins tested have different mechanisms of action, and therefore a single relevant common endpoint can hardly be chosen. Therefore, we designed a multi-parametric approach for an integrative analysis of toxic responses. To this end, we selected different endpoints among widely studied toxicological pathways.

Our study revealed that OA was cytotoxic, induced DNA strand-breaks and triggered the release of IL-8 from differentiated Caco-2 cells, similar to the effects reported with proliferative Caco-2 cells by Ferron et al. (2014). These effects correspond to observations from *in vivo* studies in rodents that reported erosion of the epithelium and release of inflammatory mediators following oral exposure to OA (Aune et al., 2012, Tubaro et al., 2003, Ito et al., 2002). PTX-2 did not affect Caco-2 cell viability and only slightly induced DNA strand-breaks at the highest concentrations. SPX-1 and YTX did not induce any effect on all tested endpoints. The absence of toxic effects correlates well with *in vivo* studies in rodents that did not report particular effects on the gastro-intestinal tract following oral exposure to these toxins (Aune et al., 2002, Tubaro et al., 2003, Munday et al., 2012). Irrespective of the phycotoxin, no ROS production was detected. However we cannot exclude that ROS might have been produced earlier and were already neutralized after 24h due to protective cellular anti-oxidant systems

Two different conceptual approaches were employed to assess the combined effects of phycotoxins in mixture. The TA method and IA concept offer the advantage to be easy to handle, fast and applicable to all kind of data. We showed that under the present settings and parameters additivity was rarely predicted irrespective of the mixture and the endpoint tested, meaning that the phycotoxins tested might be able to interact and modulate their toxic effects. Therefore, in absence of additivity, the two approaches are not appropriate to predict the toxicity of phycotoxin mixtures. In this case, the combined effects of phycotoxins must be determined from experimental data. Besides, caution needs to be taken with mixture simulations. For instance, in our study, IA analysis revealed strong synergism towards cell viability for OA/SPX-1 and OA/YTX (Figure 7) whereas raw data revealed only a slight or no increase of toxicity with mixtures (Figures 3a and 5a). It is noteworthy that a general assumption of additivity may not be reliable since OA is believed to target different serine/threonine protein phosphatases (PP) depending on its concentration: the estimated IC_{50} for PP1 is 10-100 nM, while being 100-fold inferior for PP2A (0.1-1 nM) (Cruz et al., 2013). Indeed, Gehringer (2004) suggested that the PP inhibitors microcystin-LR and OA showed a dualistic response by inducing different cellular effects depending on the concentration used, and therefore on the PP isoform inhibited.

Since few years, the Chou-Talalay method (Chou and Talalay 1984) has received considerable interest in the context of food contaminant mixtures (Alassane-Kpembi et al., 2016, Ferron et al., 2016, Smith et al., 2017, Karri et al., 2018). This easy-to-use method is a powerful tool which offers a quantitative definition for additivity and the type of interaction (synergism or antagonism) in chemical mixtures.

Based on the median-effect equation, it requires that each compound alone and in mixtures follows the principle of the mass-action law (e.g., $r > 0.95$). Therefore the method is not suitable when one drug does not exert any effect (Chou, 2006). In our study, most endpoints were not affected by at least one of the toxins, and consequently the Chou-Talalay method would not be appropriate.

Our study revealed that only OA provoked IL-8 release. However, we observed a greater effect at high concentrations with the OA/YTX mixture, meaning that YTX may potentiate the OA response. A similar hypothesis can be deduced for the cytotoxicity of OA/PTX-2 mixture. However, confirmation of such observations through a mathematical model was not possible since none exists for testing potentiation.

Irrespective of the toxin, mixtures with OA always resulted in a diminished toxic response at low concentrations (ROS production, IL-8 release and H2AX phosphorylation), whereas a higher toxic response was observed for some endpoints at high concentrations. A diminished ROS production might be linked to a diminished DNA damage response, as it is known that ROS can cause DNA breaks (Moloney and Cotter 2018). Moreover, many studies have shown that the inhibition of ROS by the use of a scavenger leads to a lowered DNA damage response (Luo et al., 2013, He et al., 2014, Chen et al., 2018). ROS have been shown to activate NF-κB, a transcription factor responsible for IL-8 regulation (Kurata et al., 1996, Pahl 1999, Oeckinghaus and Ghosh 2009). Since all these endpoints are interconnected, the systematic decrease of responses that was observed with mixtures at low concentrations in our experiments appears consistent. The protective mechanisms behind this might relate to changes in the absorption and/or the metabolism of toxins in the mixtures. For instance, OA was previously found to interact with regulatory nuclear receptors such as PXR (Fidler et al., 2012; Ferron et al., 2016), which regulate the expression of some cytochrome P450 enzymes (Wang et al., 2012). PTX-2 is believed to interact with the AhR since it induces CYP1A protein in hepatic cells (Alarcan et al., 2017). Moreover, OA, PTX-2 and SPX-1 have been shown to be metabolized through cytochromes P450 (Guo et al., 2010, Hui et al., 2012, Kittler et al., 2014, Alarcan et al., 2017). Therefore, at low concentrations, mixtures might induce CYP activity and efflux transporter expression, resulting in higher detoxification/excretion of toxins and thus decreased toxic effects. In favor of this hypothesis, the few oral *in vivo* studies conducted with phycotoxin mixtures showed a reduced uptake of toxins (Alarcan et al., 2018). As PTX-2, YTX and SPX-1 have different mechanisms of action, we assume that the potential protective effect, as suggested by the mathematical evaluation of our *in vitro* data, did not result from the disturbance of a single specific OA-dependent pathway. The strong synergistic effect observed for the cytotoxicity of the OA/PTX-2 mixture at higher concentrations may imply cytoskeleton homeostasis. Indeed, even if no cytotoxicity following treatment with PTX-2 has been observed, the toxin clearly disrupts the actin network (Alligham et al., 2007). OA has also been demonstrated to change the organization of the actin network (Fiorentini et al., 1996; Vale and Botana 2008). Disruption of the cytoskeleton has been shown to be a starting event leading to an induction of pro-apoptotic effectors (e.g caspase-3 activation) (Yamazaki et al., 2000, White et al., 2001, Martin and Leder 2001, Kulms et al., 2002). Therefore, the disruption of the actin cytoskeleton by both, PTX-2 and OA, may explain the possible synergism observed with the mixture.

Toxins of the OA group and of the PTX group share a common European regulatory limit but their behavior in mixtures is largely unknown. Results from this study suggest a possible synergistic cytotoxic effect at moderate and high concentrations in Caco-2 cells, a model mimicking the human intestinal barrier. Further research is needed to confirm the present findings and to judge on their relevance for humans exposed to phycotoxins via the diet.

5. Conclusions

In this study, we assessed the *in vitro* effects of binary mixtures of lipophilic phycotoxins on human intestinal cells towards multiple toxicity endpoints. Mainly antagonistic effects were depicted at low concentrations and, in few cases, a strong synergism was detected at higher concentrations. These findings need to be confirmed with additional experiments and completed with *in vivo* studies to bring further evidence on the combined effects of lipophilic phycotoxins.

Declarations of interest: none

Authorship Contributions

Participated in research design: Alarcan, Le Hégarat, Fessard, Hessel-Pras, Lampen

Conducted experiments: Alarcan, Barbé

Contributed new reagents or analytical tools:

Performed data analysis: Alarcan

Wrote or contributed to the writing of the manuscript: Alarcan, Le Hégarat, Kopp, Hessel-Pras, Braeuning, Lampen and Fessard

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