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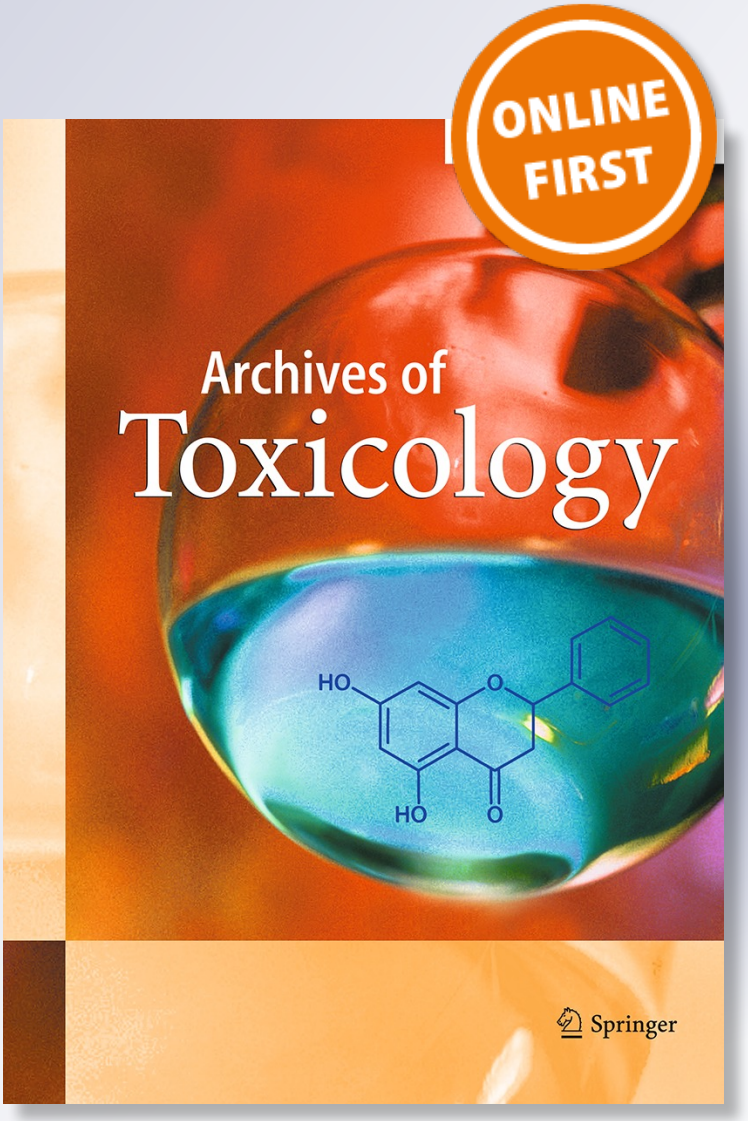

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ORGAN TOXICITY AND MECHANISMS

The emerging mycotoxin, enniatin B1, down-modulates the gastrointestinal toxicity of T-2 toxin in vitro on intestinal epithelial cells and ex vivo on intestinal explants

Martine Kolf-Clauw · Marcia Sassahara · Joelma Lucioli · Juliana Rubira-Gerez · Imourana Alassane-Kpembi · Faouzi Lyazhri · Christiane Borin · Isabelle P. Oswald

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Abstract Enniatins, the most prevalent emerging mycotoxins, represent an emerging food safety issue, because of their common co-occurrence with other fusariotoxins such as trichothecenes co-produced by *Fusarium spp* on field grains and because of their extensive prevalence in grains. In this study, the intestinal toxicity of enniatin B1 (ENN) alone and mixed with the most toxic trichothecene T-2 toxin (T2) was characterized by using two biological models from pig, the most sensitive species: the intestinal cell line IPEC1 (in vitro exposure) and jejunal explants (ex vivo exposure). Dose-dependent decreases in cell proliferation in IPEC1 and in the histopathological scores of explants were observed for ENN at μM-levels and for T2 at nM-levels, with IC50 values for ENN of 15.8 and 29.7 μM, and for T2 of 9.3 and 15.1 nM in vitro and ex vivo, respectively. Interaction analysis by probabilistic and by determinist approaches showed a less than additive effect both in vitro and ex vivo, at IC50 values, with increasing antagonism with decreasing concentrations of toxins. The results obtained by the determinist median-effect dose analysis and by the nonlinear regression analysis were concordant. All the median-effect doses estimated for IPEC cells were included in the IC50 confidence intervals of the nonlinear regression fitting. Given the occurrence of enniatins, potential synergy following the co-occurrence of enniatins and the major fusariotoxins, especially trichothecene B deoxynivalenol should be investigated.

Keywords Emerging mycotoxins · Enniatins · Gastrointestinal toxicity · Jejunal explants · Interactions · Combination index

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Introduction

The increasing world population requires more and safe food in the future, but the worldwide contamination of cereals and cereal products by mycotoxins, secondary metabolites produced by filamentous fungi, is of potential concern for human and animal health. In Europe, the contamination of cereals by *Fusarium* mycotoxins raises a worrisome problem linked to climatic changes and to co-contaminations (Van Der Fels-Klerx et al. 2012). Fusariotoxins, produced by various *Fusarium spp*, are most frequently present as mixtures (Rodrigues and Nahrer 2012). They include the well-known trichothecenes, fumonisins and zearalenone chemical groups, but also the enniatins, beauvericin, moniliformin and fusaproliferin, which are frequently referred as emerging mycotoxins (Jestoi 2008). These emerging mycotoxins are usually co-produced with the well-known fusariotoxins, but have very rarely been

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studied (review in Jestoi 2008; Santini et al. 2012). The natural co-occurrence of mycotoxins means that a better knowledge of their potential interactions must be considered in the risk assessment for human health. Both enniatin B1 and T-2 toxin can be produced by the same strains of *Fusarium* species, and their co-ocurrence has been observed in wheat, barley, oats, rye and grain-based products from Finland and Italy (Jestoi 2008). However, studies on the possible interaction between enniatins and co-occurring trichothecenes are lacking, that is, additive, synergic or antagonist toxic effects, and to date, the health risk of these combined exposures is unknown.

Enniatins represent an emerging food safety issue and possess a wide range of biological properties, but their toxicity in vivo has been little studied formerly. Oral doses of 0.5–1 g/kg body weight (bw) per day over 6 days to mice and single oral doses of up to 50 mg/kg bw in rats did not produce toxic effects (Gaümann et al. 1950; Bosch et al. 1989). Chronic exposure by feeding experiments may induce feed refusal, weight loss and reduced productivity (Jestoi 2008). In vitro, general cytotoxicity was described at low micromolar concentrations (Behm et al. 2009; Ivanova et al. 2006; Jestoi 2008). Enniatins derive structurally from amino-acids, naturally present as mixtures of cyclic depsipeptides, acting as ionophores with antibacterial activities. Among the enniatins, enniatin B1 (ENN) is a frequent contaminant in cereals, but despite this, very limited data are available on its toxicity.

The trichothecene type A T-2 toxin (T2) is recognized as the most acutely toxic trichothecene (for review, see EFSA 2011) showing high gastrointestinal sensitivity (Pinton et al. 2012a). The effects observed in various species after acute oral T2 exposure to doses ranging from 0.06 to 10 mg/kg bw include nonspecific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages and histopathological signs of necrosis of the gastrointestinal epithelium and of other target tissues (necrotoxin). T2 acute toxicity on actively dividing tissues, including intestinal mucosa, makes it a potential candidate as a biological warfare agent. Apoptosis has been demonstrated in vitro and in vivo in gastrointestinal, haematopoietic and lymphoid tissues (Doi et al. 2006), but the mechanisms involved are still controversial. The most sensitive species is the pig, and the risk assessment for consumers is based on a feeding study in this species (EFSA 2011).

The aim of the present study was to investigate how ENN modulates T2 toxicity in the situation of co-contamination, by analysing the acute toxicity of T2 on the digestive target. Two relevant models from pig were used: the IPEC cell line in culture (Bouhet et al. 2006) and pig jejunal explants (Kolf-Clauw et al. 2009). Two analytical methodologies were used and compared to study the interactions, one based on a statistical approach and the other based on a determinist approach.

Materials and methods

Toxins

Purified T2 from Sigma-Aldrich (Saint-Quentin Fallavier, France) and ENN from BioAustralis (Le Perray en Yvelines, France), were dissolved in DMSO and stored at −20 °C before dilution in cell culture media. The range of concentration of toxins in single exposure (0.3–100 μmol/L and 0.3–100 nmol/L for ENN and T2, respectively) was chosen according to previously described cytotoxic ranges and to preliminary cell assays.

In vitro assay: cell culture

IPEC-1 cells, derived from the small intestine of a newborn unsuckled piglet were maintained as previously described (Bouhet et al. 2006). Cell proliferation was used as the endpoint for a cytotoxic effect. The cytotoxicity of each toxin alone and in combination was evaluated by determining the molecular concentrations giving 50 % inhibition of cell proliferation (IC_{50}). The IC_{50} values for cells were determined for T2, ENN and the association T2 + ENN, with a constant T2:ENN ratio of 1:1,000, by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Charbonnières-les-Bains, France) according to manufacturer instructions. The mono-oxygenation of luciferin is catalysed by luciferase and depends on the presence of Mg^{2+}, ATP and molecular oxygen. The quantity of ATP is directly proportional to the number of cells. IPEC-1 cells were seeded at 4×10^3 cells/well in 100 μl of complete proliferation medium in flat-bottomed white chimney 96-well plates (Greiner, Courtaboeuf, France). After 48 h of culture, concentrations ranging from 0 to 30 μmol/L of ENN and 0–30 nmol/L of T2 were added to the cells, either separately or concomitantly (co-contamination exposure). After 48 h of treatment, 100 μl of CellTiter-Glo® Reagent were added per well and mixed for 2 min in an orbital shaker to induce cell lysis. Data were recorded 10 min after with a microplate luminometer reader (Tecan, Lyon, France) and corrected for the background signal resulting from reagent-treated medium without cells. The effect of the mycotoxins on cell proliferation was calculated as a percentage of the proliferation of the control cells without mycotoxins (relative proliferation).

Ex vivo assay: intestinal explant culture

Six crossbreed 4–5 week-old weaning piglets were used to obtain explants of jejunal tissue and the procedures for the culture of the explants were as previously described (Kolf-Clauw et al. 2009) with minor modifications. Explants were incubated for 4 h in William's Medium E (WME)

containing 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin and supplemented with D glucose (2.5 g/L) and 30 mM Alanine-Glutamine (Sigma) at 37 °C under CO_2-controlled atmosphere with orbital shaking. Uncultured control tissue was placed into fixative at the end of dissection time (0 h). Explants were exposed to purified T2 at 0, 0.3, 1, 3, 10 and 30 nM, or purified ENN at 0, 0.3, 1, 3, 10 and 30 μM, or to the mixture of T2:ENN at the same ratio as for cell culture (1:1,000) for 4 h. For histological analysis, jejunum explants were fixed in 10 % buffered formalin for 24 h. Histopathological and morphological assessment of the explants were carried out as previously described (Kolf-Clauw et al. 2009).

Data analysis: probabilistic and determinist approaches

For data analysis, two toxin-based approaches by response-additivity modelling (Berenbaum 1985) were used and compared: one statistically based analysis, with regression curve fitting and *P* value determination and the other based on determinist analysis.

Statistical analysis

Firstly, the individual dose–effect data were analysed to verify that both drugs produced effects that increased with dose. For each dose level, the percentage of proliferative cells compared to controls, and the explant scores are presented, as mean ± SD of 5–6 independent experiments (from different animals for the intestinal explants). To test the individual and combined effect of the toxins according to the concentration, ANOVA analysis was used (Systat Software Inc, version 10, San Jose, USA) followed by Tukey and Dunnet tests if significantly different (P values ≤ 0.05). The IC_{50} values of each mycotoxin alone and of the mixture were calculated by extrapolating results from the concentration–response curves from each experiment, by nonlinear regression Hillslope modelling using the raw data (GraphPad Prism5 software Inc, San Diego, California, USA). To test the interactive effect, an isobologram approach was used, with the assumption of a constant potency ratio of ENN/T2, employing the concept of dose equivalence (Tallarida 2006). A constant potency ratio of 1,000:1 (ENN:T2) was used, and additive isobolograms represented by a line joining equally effective doses were constructed. The cytotoxic effect was identified as synergist, additive or antagonist according to the position of the combined effect related to the line of additivity (Borgert et al. 2001).

Median-effect doses and combination index

The second approach was based on a determinist analysis with the determination of combination index values (CI) by the Chou-Talalay median-effect equation (Chou and Talalay 1984). The median-effect plot of Chou was applied to the individual toxins, and a median-proliferation dose (Dm) was calculated for each toxin (CompuSyn Software 2007) and compared with the IC_{50} values calculated by the regression model. For the mixture of T2 and ENN, the Dm for the mixture was calculated. The combination index (CI) was calculated for a 10, 20 and 50 % inhibition of cell proliferation. CI is a quantitative measure of the degree of drug interaction of synergism and antagonism for a given endpoint of the effect measurement with $CI < 1$ indicating synergism, that is, a greater than expected additive effect and $CI > 1$ indicating antagonism, a smaller than expected additive effect.

Results

Individual and combined effects of the ENN and T2 on intestinal epithelial cell proliferation

Each treatment, ENN (0.3–100 μM), T2 (0.3–100 nM) and ENN+ T-2 induced a dose-dependent decrease in intestinal epithelial cell proliferation ($P < 0.001$). ENN significantly decreased cell proliferation at all the concentrations tested (0.3–100 μM), with values ranging from 96.2 to 13.6 % proliferation when compared to control cells. T2 also decreased cell proliferation, from concentration of 3 nM and above, with relative cell proliferation of 80 % decreasing to 11.6 % at the highest concentration (Fig. 1). The IC_{50} values were 15.80 μM (confidence interval of 13.02–19.17 μM) and 9.35 nM (confidence interval of 6.94–12.6 nM) for ENN and T2, respectively (Table 1). The binary mixture of T2 and ENN at the 1:1,000 ratio showed a dose-dependent effect on cell proliferation, ranging from 96.3 to 3.1 % compared to the control cells (Fig. 1), and an IC_{50} of 14.41 μM (confidence interval of 10.55–19.67). The determination coefficients of the nonlinear regression Hillslope showed a better goodness of fit for isolated toxins than for the mixture, with values of 0.995 for enniatin, 0.989 for T2-toxin and 0.840 for the mixture (Table 1). The median-effect doses (Dm) calculated according to the determinist method were highly correlated to the IC_{50} values, and the coefficients of correlation of the median-effect plot were between 0.925 and 0.997 (Table 1).

Individual and combined effects of the toxins on jejunal explant histopathology

Each treatment, ENN (0.3–30 μM), T2 (0.3–30 nM) and ENN + T2 at similar concentrations as the toxins alone induced a dose-dependent decrease in the histopathological scores of the jejunal explants after 4 h of exposure

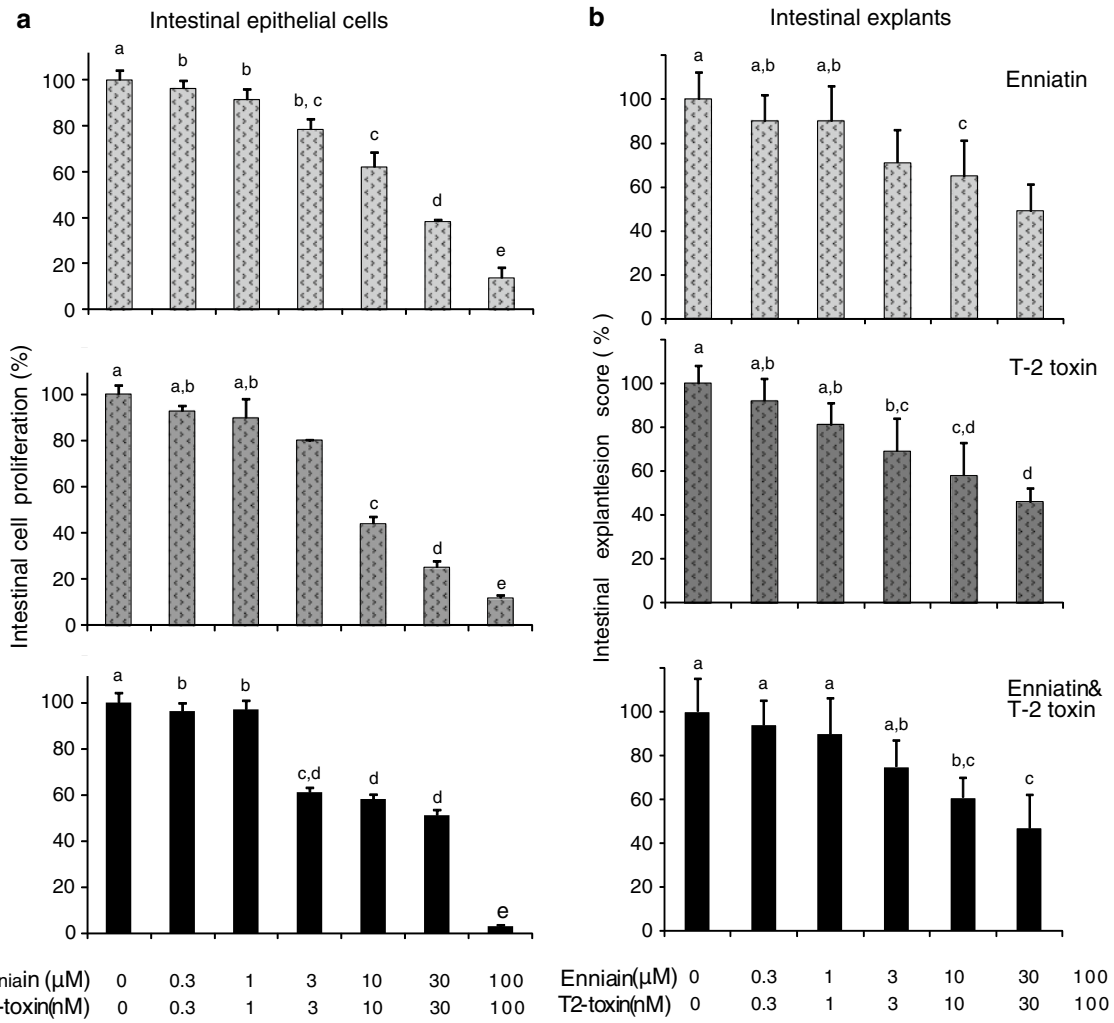

Fig. 1 **a** Effects of mycotoxins on the proliferation of IPEC cell cultures after 48-h exposure to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2-toxin 1,000:1). Mean relative proliferation compared to control cells (expressed as %) and the standard deviation (SD) of the mean. ANOVA analysis, *bars* without a common *letters* differ ($P < 0.05$). **b** Jejunal explants from 4 to 5 week-old piglets were exposed in vitro for 4 h to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2 1,000:1) before histopathological examination and scoring assessment. For each concentration, 2–4 explants from the same animal were scored. Data are mean relative scores ± SD from 6 different animals (expressed as %). ANOVA analysis, *bars* without a common *letters* differ ($P < 0.05$)

Table 1 IC50 and median-effect dose (Dm) in IPEC cell culture (Intestinal Porcine Epithelial Cell) and in porcine jejunal explants culture, following exposure to mycotoxins alone or in combination

	Enniatin B1 µM		T-2 toxin nM		T-2 toxin + Enniatin B1 (1:1,000) µM	
	IC50	Dm	IC50	Dm	IC50	Dm
IPEC	15.80 (13.02–19.17) $R^2 = 0.995$	14.97 ($r = 0.997$)	9.35 (6.94–12.60) $R^2 = 0.989$	9.34 ($r = 0.985$)	14.41 µM (10.55–19.67) $R^2 = 0.840$	10.5 ($r = 0.925$)
Jejunum explants	29.71 $R^2 = 0.60$	28.32 ($r = 0.959$)	15.11 $R^2 = 0.60$	18.08 ($r = 0.987$)	18.58 $R^2 = 0.787$	18.76 ($r = 0.993$)

IC50 values obtained using the Hillslope model (confidence interval of IC50)

Dm, Median-effect dose calculated by the Chou-Talalay median-effect equation: determinist approach; *r* coefficient of correlation; R^2 determination coefficient of the nonlinear regression model

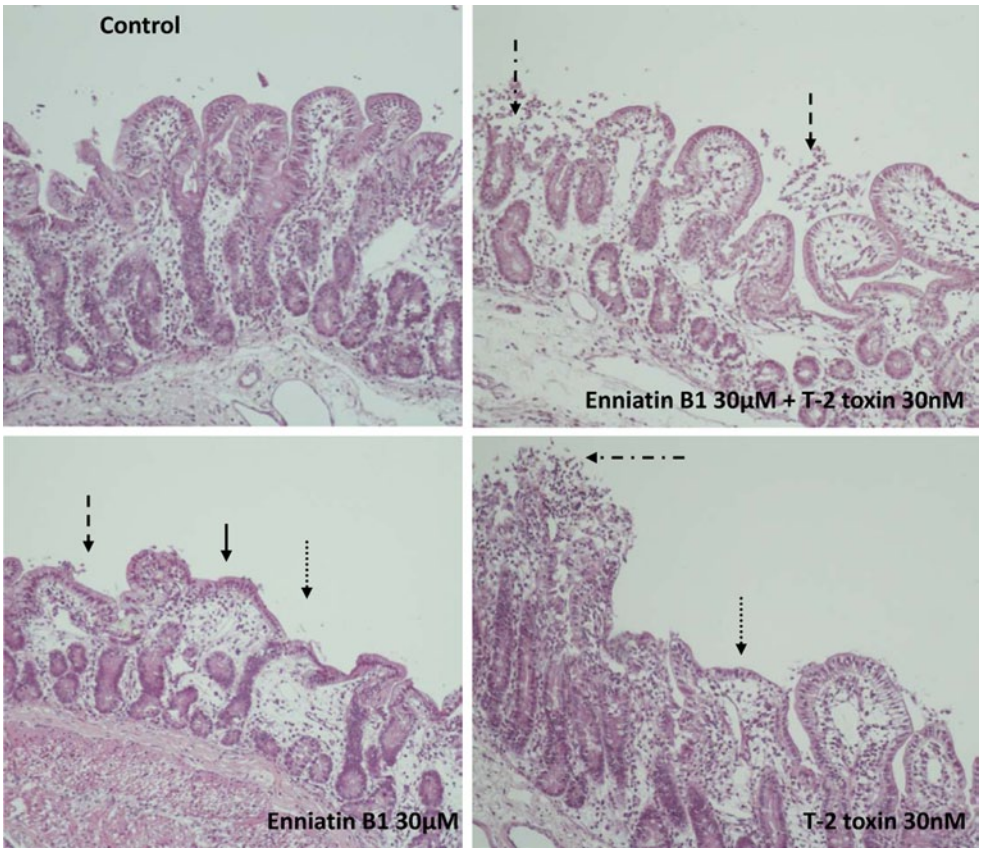

Fig. 2 Morphology of 4–5 week-old piglet jejunal explants incubated for 4 h with T-2 toxin, Enniatin B1 and the binary mixture of Enniatin B1 and T-2 toxin (1,000:1). Negative control (*upper left*) and treated samples with indication of lesions observed for the scoring system analysis. Obj 10x, H&E staining. ——▶ Coalescence, - - - - -▶ Cellular debris, ...▶ Cuboïd cells, -·-·-·▶ Villi lysis

($P < 0.001$). The morphological changes consisted of coalescent villi with cubic epithelial cells instead of the cylindric epithelial cells seen in the control. Lesions included lysis of villi, areas of oedema in the lamina propria and cellular debris (Fig. 2). The morphological and lesional scores were significantly decreased compared to the control explants from the concentrations of 3 µM ENN, 3 nM T2 and the combination of both toxins at 10 µM/10 nM (ENN/T2, 1,000:1). The corresponding scores were reduced to about 70 % of the control explants at 3 nM T2 or 3 µM ENN (and to almost three-quarters of the control values for the similar points in the mixture) to less than half the mean score (45.7–48.9 %) of control explants at the highest concentrations (Fig. 1). The IC_{50} values could not be precisely estimated in the case of explants as the 95 % confidence intervals were too large to test the interaction by the classical isobologram approach. However, the nonlinear fitting of the raw data enabled the $IC_{50.}$ values to be obtained: 29.71 µM for enniatin, 15.11 nM for T2-toxin and 18.58 µM for the mixture (Table 1). These values are very similar to and concordant with those obtained by the determinist approach with Dm of 28.32 M, 18.08nM and 18.76 µM for ENN, T2 and the mixture, respectively. The correlation coefficients of the median-effect plot were between 0.959 and 0.993 for the explants model.

Determination of the type of interaction between T2 and ENN at the intestinal level

A less than additive effect was observed in the two biological models by both methodologies for the mixture ENN/T2 (1,000:1). The two mathematical approaches resulted in similar results. In the determinist approach, the goodness of fit was attested by coefficients of correlation higher than 0.925 for the modelization using the two biological models (Table 1). The comparison of Dm and IC_{50} shows highly correlated values (Table 1). The Chou and Talalay method does not allow a confidence interval to be calculated with all the raw data, but we show here that all the calculated Dm for the IPEC cell culture model were included in the IC_{50} Hillslope confidence intervals. At the Dm, that is, 50 % effect level, the CI were 1.81 and 1.69 for IPEC and explants, respectively, indicating antagonism. CI values at doses lower than the Dm, more relevant for risk assessment purposes, were estimated for the 10, 20 and 30 % effect levels. Antagonism, that is, a less than additive effect, was also observed for these effect levels (Fig. 3).

The less than additive effect was demonstrated in both biological models, with the CI index calculated in both models, IPEC cells or jejunal explants giving very similar

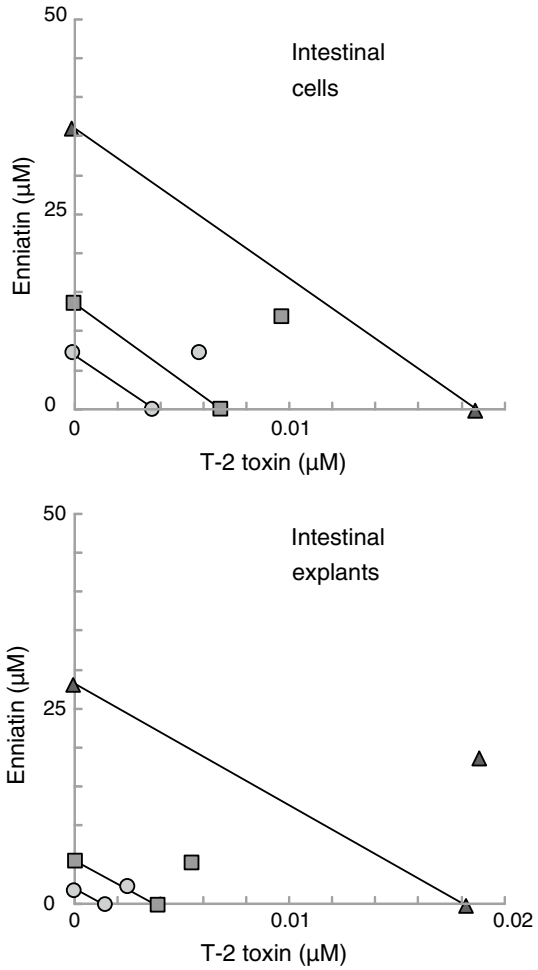

Fig. 3 Isobolograms illustrating the combined effect of the mixture of Enniatin B1 and T-2 toxin (1,000:1) for reaching 20 % (Fa = 0.2, *filled circle*), 30 % (Fa = 0.3, *filled square*), or 50 % inhibition (Fa = 0.5, *filled triangle*) of IPEC cell proliferation or of the scores of explants. The points on each axis are mean concentrations of dose–response curves of each toxin alone (*Compusyn*® software analysis)

values and well correlated: $r = 0.98$ (Table 2). In both models, the binary mixture of the toxins produced antagonism, tending towards strong antagonism with decreasing the effect (strong antagonism according to Chou 2006, means a CI between 3.3 and 10). The two alternative models gave similar and highly correlated results for moderate effects, that is, less than the median-effect that is biologically relevant. Above the effect dose of 95 % inhibition for cell relative proliferation and for the relative score of explants, CI values were lower than 0.90, indicating synergy. However, from a biological and toxicological point of view, this result should not be considered as relevant, because mixture interaction should be identified at non-toxic levels that are relevant and realistic in terms of the actual exposure of consumers.

Table 2 Combination index (CI) values in the two models

Effect level	10 %	20 %	30 %	50 %
IPEC cells	3.26	2.63	2.27	1.81
Jejunum explants	4.10	2.96	2.38	1.69

Combination index (CI) calculated by the Chou-Talalay median-effect equation (1984) by using CompuSyn software (3.0.1, 2010) in the two pig models: intestinal IPEC-1 cells (in vitro model) and jejunal explants (ex vivo model). Effect levels for 10, 20, 30 and 50 % of the relative proliferation for treated cells compared to control cells, or the relative scores for explants. CI is a quantitative measure of toxin interaction in terms of synergism and antagonism for a given endpoint of the effect measurement. CI = 1 means additive effect, combined effect predicted by the mass-action law principle in the absence of synergism (CI < 1: greater than expected additive effect) or antagonism (CI > 1: smaller than expected additive effect)

Discussion

Both fusariotoxins, T2 and ENN individually induced dose-dependent gastrointestinal toxicity with a one-thousand-fold difference in potency, when assessed on an intestinal cell line (IPEC) and on intestinal explants. The ratio in the cytotoxic effect between ENN and T2 (1,000:1) was similar to other studies (Behm et al. 2012). In combination, these toxins showed a less than additive effect in the two biological models. To our knowledge, no previous interaction study has used two different biological models of different complexity, at the cellular level for the IPEC cell line and at the tissue level for jejunal explants. These two intestinal models were from pig, considered the most relevant animal species for studying fusariotoxin toxicity (Pinton et al. 2012a). The mycotoxins T2 and ENN were evaluated because of their special interest and natural co-occurrence. T2 is of concern as the most acutely toxic member of the trichothecenes. Our results confirm the high toxicity of T2 towards rapidly dividing cells such as mammalian kidney epithelium (Ruiz et al. 2011b) or in human monocytes (Hymery et al. 2006). The digestive tract as a target is well-known for T2, with the induction of necrotic lesions of the gastrointestinal tract following high doses in several species (Pinton et al. 2012a). In the present study, the co-contamination with ENN down-modulated this toxicity. Identifying intestinal target is of major importance because the intestinal epithelium represents the first barrier to the access of food contaminants or pathogens to the whole body. Its integrity is also necessary to avoid any indirect effects of food contaminants. More recently, another fusariotoxin, deoxynivalenol, has been shown to alter the intestinal epithelium in vitro, ex vivo and in vivo (Bracarense et al. 2011; Kolf-Clauw et al. 2009; Pinton et al. 2009). The morphological changes observed in jejunal explants with T2 or ENN in the current study were similar to those

described with deoxynivalenol (Kolf-Clauw et al. 2009; Pinton et al. 2012b). Studying the toxicity of the enniatins alone and in combination, in situ on the digestive target is especially relevant.

ENN is an emerging fusariotoxin, which has led to interest concerning its toxicity over the last few years. Even though enniatins apparently are of low acute toxicity in vivo, their effects in combination with other mycotoxins remain unknown. They represent an emerging food safety issue because of their extensive incidence, documented in recent decades, in various grain cereals. In a Spanish survey, the simultaneous presence of two or more mycotoxins was observed in a high percentage of the samples, and the prevalence of the emerging mycotoxins in cereal products was considered likely to pose a health risk to general population (Serrano et al. 2013). Our cytotoxicity data for ENN are in accordance with literature data reporting enniatin cytotoxicity (Jestoi 2008). The measured IC50 values were similar to those reported in Caco-2 cells (Meca et al. 2012) and were in agreement with other studies reporting IC50 values in the lower μM-range (Föllmann et al. 2009; Ivanova et al. 2006), with a higher sensitivity for cancer cells, possibly making enniatins interesting as new anticancer drugs (Wätjen et al. 2009). Recently, the cytotoxicity of enniatins was shown to be due to lysosomal destabilization and mitochondrial permeabilization, possibly related to their ionophoric properties, finally resulting in apoptotic cell death (Gammelsrud et al. 2012; Ivanova et al. 2012).

Co-contaminations are frequent in cereals in Europe and worldwide (Rodrigues and Naehrer 2012) and studying combined toxicity of mycotoxins should help to protect consumers. The simultaneous appearance of T-2 toxin and enniatin B1 in the same samples can be inferred by the recent multi-mycotoxin analysis study of 83 feed samples (Streit et al. 2013). The chosen combinations were realistic, corresponding to some actual situations in food or feed, as T2 and ENN concentrations have been reported in the μg/kg (Van der Fels-Klerx and Stratakou 2010) and in the mg/kg (Santini et al. 2012; Uhlig et al. 2006) ranges, respectively. In our study, two different analytical models were used that gave concordant results for the interaction. The T2 and ENN interaction was analysed according to a classical probabilistic approach, and also with a determinist method (Chou and Talalay 1984). This latter method provides a fundamental basis for assessing whether a combined effect is greater, equal or smaller than expected effect. This method was initially described for optimizing drug associations for pharmacologists (Chou 2006) and has subsequently been applied to toxicology studies of mycotoxins (Ruiz et al. 2011a, b). The present results from determinist analysis were correlated with the probabilistic results in the two biological models. For cell cytotoxicity, all the median-doses estimated by the determinist method were included in the confidence intervals of Hillslope model, demonstrating a good concordance with the probabilistic approach. The Chou and Talalay method enables the nature of an interaction at various dose levels to be easily predicted. Interactions previously described between co-occurring mycotoxins showed either additive affects, antagonism or synergism, according to the biological model, the toxins, and the level of the effect. The combination of the two major trichothecenes T2 and DON was previously reported as antagonist in kidney cells, and similarly to our results, the strongest antagonism on cytotoxicity was observed at lower doses (Ruiz et al. 2011b). These latter authors used Chou and Talalay's method to analyse the fractional inhibition of proliferation by beauvericin, DON and T-2 toxin in combination. Strong antagonism was also observed with T-2 toxin and the emerging mycotoxin beauvericin, as for the IPEC cells and jejunal explants with ENN/T2 in our study. We observed stronger antagonism below 50 % relative proliferation inhibition in the two biological models, but interactions tending towards synergism at higher levels of the effects. Testing a combination at low concentrations appears highly relevant biologically compared to testing at high concentrations, such as above 75 % inhibition for cell relative proliferation as described previously (Ruiz et al. 2011b). We have to wonder about the relevance of the mechanisms underlying these interactions observed at high toxic concentration, whereas interactions at very low levels of effects are very relevant, because based on healthy cell metabolism and reactivity, allowing hypothesis relative to the mechanisms of these interactions.

Biologically, several hypotheses could be proposed to explain the less than additive effect of the two toxins observed in vitro and ex vivo on the digestive tract. First, antagonism may be explained by the interactions at membrane level, with ENN potentially lowering the cell bioavailability of T2. Indeed, the ionophoric properties of ENN might interact with the cell membrane, preventing T2 from reaching its cell target. Another hypothesis at membrane level is an interaction involving an efflux pump and preventing the molecular target to be reached, as enniatins interact with ATP-binding cassette transporters below toxic concentrations (Ivanova et al. 2010; Tedjiotsop Feudjio et al. 2010). Enzymatic inhibition of HT2 production is another hypothesis, as well as an antagonism at the intracellular target level. T-2 toxin was shown to induce apoptosis in the intestinal crypt epithelial cells in mice (Li et al. 1997) that correlated in vitro with MAPK activation (Yang et al. 2000). A target antagonism could explain, at least partly, our results at the level of MAP-Kinases, as T2 activates extracellular regulated protein kinases ERK (ERK1/2, Yang et al. 2000), whereas enniatin B1 was recently shown to decrease the activation of ERK

(Wätjen et al. 2009). These different hypotheses should be investigated to explain the observed interactions between ENN and T2.

To conclude, the present study demonstrates the down-modulation of the gastrointestinal toxicity of T-2 toxin by the emerging enniatin B1 below toxic concentrations and confirms the relevance of the determinist approach for the analysis of toxin interactions, giving concordant results with the probabilistic approach in two different pig intestinal models. Further toxicity studies are needed with the emerging mycotoxins, notably investigations into the interactions following co-occurrence of enniatins A1 and B1 and major fusariotoxins, especially the trichothecene B deoxynivalenol. In addition, the potential bioaccumulation in animal and human tissues of the enniatins due to their lipophilicity is a matter of concern.

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