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IN VITRO SYSTEMS

Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetylated derivatives in intestinal epithelial cells

Imourana Alassane-Kpembi · Olivier Puel · Isabelle P. Oswald

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Abstract In case of mycotoxin contaminations, food and feedstuff are usually contaminated by more than one toxin. However toxicological data concerning the effects of mycotoxin combinations are sparse. The intestinal epithelium is the first barrier against food contaminants and this constantly renewing organ is particularly sensitive to mycotoxins. The aim of this study was to investigate the effects of deoxynivalenol (DON) and four other type B trichothecenes (TCTB), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon-X (FX) alone or in combination on intestinal epithelial cells. Proliferating, non-transformed IPEC-1 cells were exposed to increasing doses of TCTB, alone or in binary mixtures and mycotoxin-induced cytotoxicity was measured with MTT test. The toxicological interactions were assessed using the isobologram-Combination index method. The five tested mycotoxins and their mixtures had a dose-dependent effect on the proliferating enterocytes. DON–NIV, DON–15-ADON and 15-ADON–3-ADON combinations were synergistic, with magnitude of synergy for 10 % cytotoxicity ranging from 2 to 7. The association between DON and 3-ADON also demonstrated a synergy but only at high doses, at lower doses antagonism was noted. Additivity was observed between NIV and FX, and antagonism between DON and FX. These results indicate that the simultaneous presence of mycotoxins in food commodities and diet may be more toxic than predicted from the mycotoxins alone. This synergy should be taken into account considering the frequent co-occurrence of TCTB in the diet.

Keywords Combination index · Cytotoxicity · Non-transformed intestinal cell · Mycotoxin · Synergy · Trichothecenes

Abbreviations

3-ADON	3-Acetyldeoxynivalenol
15-ADON	15-Acetyldeoxynivalenol
CI	Combination index
DON	Deoxynivalenol
D_m	Median effect dose
DRI	Dose reduction index
f_a	Fraction affected
FX	Fusarenon-X
IC_{50}	Inhibitory concentration 50 %
MTT	3,(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide
NIV	Nivalenol

Introduction

Trichothecenes are fungal secondary metabolites produced by genera including *Fusarium*, *Myrothecium*, *Spicellum*,

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I. Alassane-Kpembi · O. Puel · I. P. Oswald (✉)
UMR 1331 Toxalim, Research Center in Food Toxicology, INRA, 180 chemin de Tournefeuille, BP93173, 31027 Toulouse cedex 03, France
e-mail: Isabelle.Oswald@toulouse.inra.fr

I. Alassane-Kpembi · O. Puel · I. P. Oswald
INP, UMR 1331, Toxalim, Université de Toulouse, 31076 Toulouse, France

I. Alassane-Kpembi
Hôpital d'Instruction des Armées, Camp Guézo, 01BP517, Cotonou, Bénin

Stachybotrys, *Cephalosporium*, *Trichoderma* and *Trichothecium* (McCormick et al. 2013). They represent a unique class of mycotoxins that do not only exert toxicity in animal but also are virulence factors in plant disease, which make them one of the major groups causing significant economic and health impacts (Desjardins 2009). The trichothecene-producing genera are adapted for colonization and growth on substrates with a wide range of moisture availability and nutrient content. An outcome of that feature is that these mycotoxins may be either food-borne contaminants or indoor environmental contaminants (Pestka et al. 2008). The adverse health effects of trichothecenes include emesis, nausea, anorexia, growth retardation, hemorrhagic lesions, neuroendocrine changes and immunosuppression (Larsen et al. 2004; Pestka et al. 2004). At the molecular level, trichothecenes display multiple inhibitory effects on primary metabolism of eukaryotic cells including inhibition of protein as well as DNA and RNA synthesis, and their activity may eventually produce harmful levels of oxidative stress due to generation of free radicals (Arunachalam and Doohan 2013). Thus organs and biological functions involving actively dividing cells appear more sensitive to this class of mycotoxins (Parent-Massin 2004; Pestka et al. 2004). The intestinal epithelium is the most vigorously renewing organ in mammals, which makes it a sensitive target to the high concentrations of trichothecenes it may be exposed to, as the first barrier against food contaminants (Maresca 2013).

Type B trichothecenes are produced by *Fusarium* species responsible of *Fusarium* head blight. Due to a high prevalence of this disease, type B trichothecenes are the most common contaminants of cereal grains in temperate regions of the world. A large scale data survey indicates that deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), fusarenon X (FX; 4-acetylnivalenol) and 3-acetyldeoxynivalenol (3-ADON) (Online Resource 1) were present in 57, 20, 16, 10 and 8 %, respectively of food samples collected in the European Union (SCOOP 2003). *Fusarium* species spoiling food and feed matrices may also produce several mycotoxins simultaneously, especially DON, NIV and their acetyl derivatives. Several studies have shown these trichothecenes to co-occur in corn, wheat and barley (Eckard et al. 2011; Schollenberger et al. 2012; De Boevre et al. 2013).

Because of their natural co-occurrence, there is increasing concern about the hazard of exposure to mycotoxin mixtures. Unfortunately, the toxicity of mycotoxins when present together, cannot always be predicted based upon their individual toxicities (Speijers and Speijers 2004). Multi-exposure may lead to additive, synergistic or antagonistic toxic effects. At the present very little is known about the actual combined health risk from exposure to mycotoxins. The aim of the present study was to assess the combined effects of the five major type B trichothecenes present in food and feed (DON, 3-ADON, 15-ADON, NIV and FX) in terms of additive, antagonistic or synergistic toxicity towards the intestinal epithelium. Pig is considered as both a target and model species to study the toxicity of mycotoxins (Ireland et al. 2008; Meissonnier et al. 2007). Indeed, due to its cereal rich diet, pig can be exposed to high concentration of type B trichothecenes. In addition, because of its similarity, the pig can be regarded as a good model of extrapolation to human. In the present study, the non-transformed porcine intestinal cell line, IPEC-1 was used to analyze the combined toxicity of type B trichothecenes.

Materials and methods

Toxins

DON, 3-ADON and 15-ADON were purchased from Sigma (St Quentin Fallavier, France). They were dissolved in dimethylsulfoxide (DMSO) to 15 mM, except 3-ADON that was dissolved to 40 mM. NIV and FX were purchased from Waco Pure Chemical Industries LTD (Osaka, Japan) and dissolved in DMSO to 10 mM. Stock solutions were stored at −20 °C and working dilutions were prepared in cell culture medium.

Final concentration of 0.6 % DMSO corresponding to the highest DMSO concentration of working dilutions was tested and results were not significantly different from controls.

Cell culture and cytotoxicity assay

The IPEC-1 cell line is a non-transformed intestinal epithelial cell line that was derived from the small intestine of a newborn unsuckled piglet (Gonzalez-Vallina et al. 1996). This cell line was a generous gift from Drs. H. M. Berschneider and D. D. Black. The IPEC-1 cells were maintained as previously described (Pinton et al. 2010). In brief, the IPEC-1 cells were grown at pig physiological internal body temperature (39 °C), 5 % CO_2, in complete DMEM/F-12 medium (Eurobio) supplemented with antibiotics, 5 % FBS, 2 mM L-glutamine, epidermal growth factor (5 µg/l; Becton–Dickinson, Le Pont de Claix, France), and ITS solution (insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml)). Before reaching confluence, the cells were trypsinized and plated at a density of 10,000 cells per well in culture medium in 96-well flat-bottom cell culture plates (Costar, Cambridge, MA, USA).

To assess the individual and combined effects of DON, NIV and their acetyl derivatives, the IPEC-1 cells seeded in 96-well plates were incubated 24 h at 39 °C,

in complete DMEM/F12 media. Then the cells were exposed for 24 h to serial dilutions of single or combination of mycotoxins or vehicle (DMSO) alone. Mycotoxin solutions were diluted in serum-free medium and the final toxin concentrations tested in the wells ranged from 0.12 µM to 150 µM. The tested ratios were 1:1 for DON–15-ADON, 1:10 for DON–3-ADON, 1:1 for DON–NIV, 1:0.8 for DON–FX and 1:0.8 for NIV–FX. These ratios, calculated from preliminary individual cytotoxicity experiments, allowed obtaining a roughly similar toxicity for each mycotoxin. As described by Gauthier et al. (2012), the cell viability after exposition to mycotoxins was assessed by means of the CellTiter 96® Non-Radioactive Cell Proliferation Assay MTT (Promega, Charbonnière, France), which is based on the cellular conversion of (3,(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide into formazan product. Briefly 15 µl of MTT solution (CellTiter 96® Non-Radioactive Cell Proliferation Assay MTT, Promega, France) was added per well before a 4-h incubation. One hundred µL of a solubilization/stop Mix (Promega, France) was then added per well, and after an additional hour room temperature incubation, the optical density (OD) was red using a multiplate reader (Infinite M200, TECAN, Austria) at 570 nm.

The percentage of viable cells was calculated using the blank-corrected OD values in the following formula:

$$\text{Viability (\%)} = 100 \times \frac{\text{Mean OD in mycotoxin(s) treated samples}}{\text{Mean OD in vehicle treated samples}}$$

Data analysis

Median-effect and combination index-isobologram analysis of mycotoxin mixtures

Dose–effect studies for single mycotoxins, and binary associations were carried out simultaneously (Fig. 2). The dose–effect relationships of the individual and combined mycotoxins were biometrically modeled by using the Median-Effect Equation of the Mass Action Law (Chou 2006):

$$f_a/f_u = (D/D_m)^m$$

where D is the dose of the toxin, f_a is the fraction affected by D (e.g. percentage of inhibition/100), and f_u is the fraction unaffected (i.e. $f_u = 1 - f_a$). D_m is the median-effect dose (e.g. IC_{50}), and m is the coefficient signifying the shape of the dose–effect relationship ($m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose–effect curves, respectively). We verified that the linear regression correlation coefficients of the median effect plots were greater or equal to 0.95 (Chou 2010).

Mycotoxin interactions were analyzed by the isobologram and combination-index (CI) methods derived from the Median-effect principle of the Mass-action Law (Chou 2006).

Regardless of the mechanisms or the units of the drugs, CI = 0.9–1.1, CI < 0.9, and CI > 1.1 indicate an additive effect, a synergism, and an antagonism, respectively. For all binary mycotoxin combinations, CI values were generated over a range of fractions of cell viability affected (f_a) from 0.05–0.95 (5–95 % toxicity). When synergy occurred in the effects of the mixtures, dose reduction indices (DRI) were calculated (Chou 2006). The dose reduction index (DRI) measures how many folds the dose of each mycotoxin in a synergistic combination may be reduced at a given effect level compared with the doses of each mycotoxin alone.

Statistical analysis

The reported values are the mean ± standard deviation (SD) of at least three independent experiments, each with triplicate wells per dose level. Statistical analyses were performed using Sigma Plot version 11.0 (Systat Software Inc., San Jose, CA, USA). Differences between all the mycotoxin treatments were analyzed by the non-parametric Kruskal–Wallis One Way Analysis of Variance on Ranks with a critical level of significance set up at $p < 0.001$. Significant differences between groups were analyzed with the Holm-Sidak post hoc multiple comparison procedure. The level of $p < 0.05$ was considered statistically significant.

The dose–effect relationship analysis for individual mycotoxin cytotoxicity, combination index (CI) and their 95 % confidence intervals calculation, the dose reduction index calculation, isobologram plots and f_a-CI plots for combined effects were all performed with Compusyn software version 3.0.1 (ComboSyn Inc., Paramus, NJ, USA).

Results

Individual and combined cytotoxicity of type B trichothecenes on IPEC-1 cells

First, we evaluated the cytotoxic effects of individual and combined type B trichothecenes on non-transformed porcine intestinal epithelial cells. The mycotoxins and their mixtures clearly showed a dose-dependent toxicity toward proliferating IPEC-1 cells (Fig. 1, Online Resource 2). The highest concentrations tested for the single mycotoxins and mixtures decreased cell viability by almost 80 % compared to control. For all data points tested, mixtures showed higher inhibition of viability than individual mycotoxins. The parameters (D_m, m, r) of the dose–effect relationships for the toxicity of the tested mycotoxins are presented in Table 1. All the dose–effect experiments showed good linear correlation coefficients of the Median-Effect plots

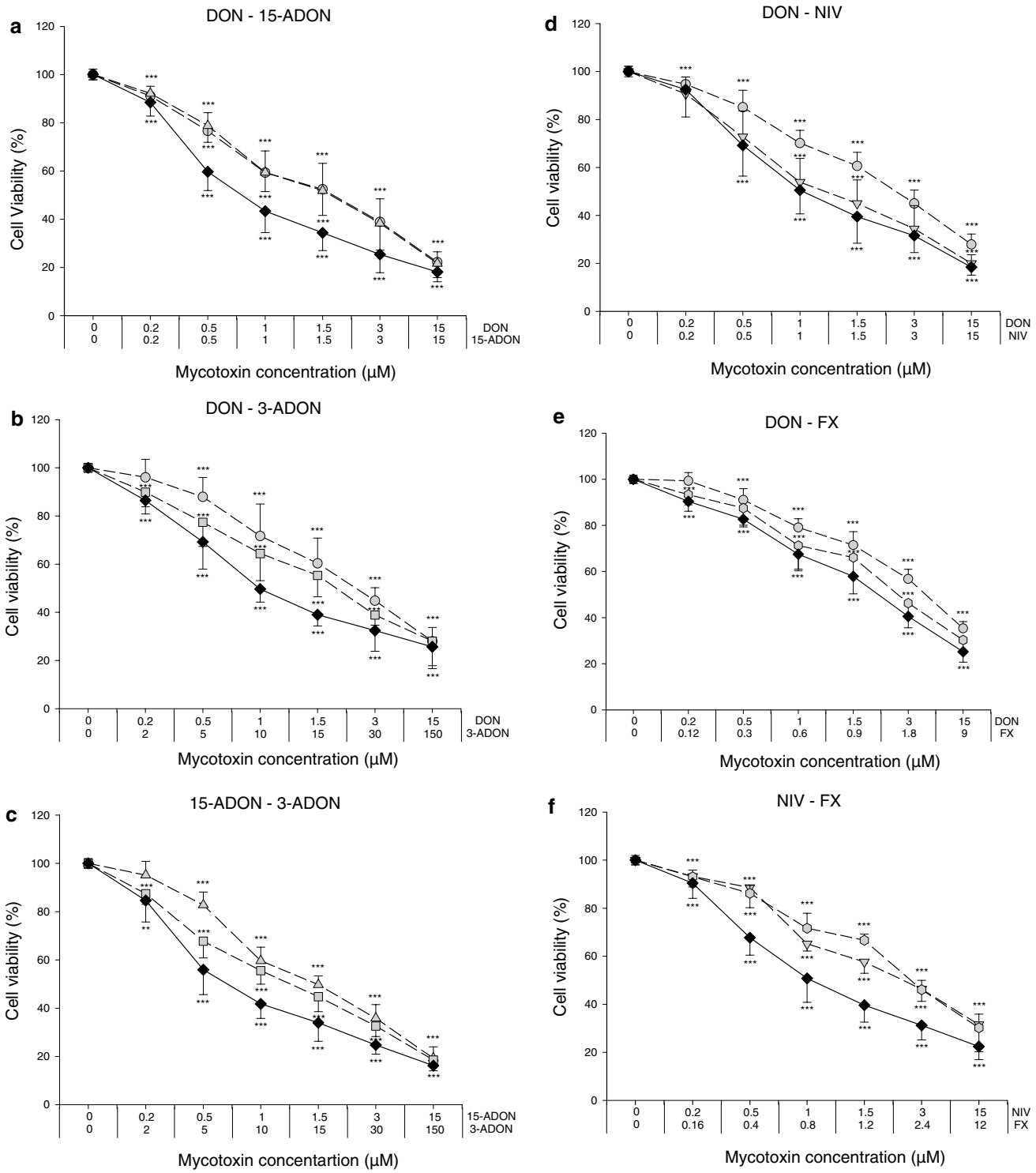

Fig. 1 Comparative toxicity of single mycotoxins DON (*circle*), 3-ADON (*square*), 15-ADON (*triangle*), NIV (*inverted triangle*) or FX (*hexagon*) and binary combinations (*diamond*) on proliferating IPEC-1 cells. Intestinal epithelial cells were exposed for 24 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the MTT assay. Data are mean ± SD of three independent experiments

Table 1 Dose-effect relationship parameters for viability inhibition by DON, NIV, and their acetyl derivatives in IPEC-1 cells

Mycotoxin	Dose-effect parameters				
	D_m (μM)	*m*	*r*	EC_{10} (μM)	EC_{80} (μM)
DON	3.56	0.90	0.974	0.31	16.54
3-ADON	19.84	0.75	0.973	1.06	125.72
15-ADON	2.41	0.94	0.967	0.24	10.47
NIV	2.11	0.87	0.975	0.17	10.34
FX	2.29	0.96	0.968	0.23	9.64

m, D_m and *r* are the slope, the median-effect dose, and the coefficient of linear correlation of the experimental data to the mass-action law. EC_{10} and EC_{80} are the effective concentrations inhibiting cell viability by 10 and 80 %, respectively

($r > 0.95$), which qualifies for further analysis using the Median-Effect principle. The D_m values for individual toxins DON, 15-ADON, NIV and FX were in the same range, while 3-ADON had roughly a ten-fold higher D_m which means it was ten-fold less potent. The analysis of the types of interaction was done by drawing isobolograms, and calculating CI values.

Synergistic associations of type B trichothecenes

The isobologram for the combination of DON and 15-ADON at three different cytotoxicity levels (10, 30 and 50 %) is presented in Online Resource 3 and revealed a synergy between DON and 15-ADON. In this type of graph, the additive combined effect follows the diagonal line between the effective concentrations of each single toxin. If the measured combined effect of two toxins is above or below the diagonal line, it indicates an antagonistic or a synergistic effect of the combination respectively. The CI values were also calculated for a wide range of cytotoxity levels.

For mixtures of DON, NIV and acetyl derivatives, the evolution of CI values for graded levels of cytotoxicity is presented in Fig. 2. Along the graded cytotoxicity levels, the DON–15-ADON combination mainly demonstrated synergy, with the higher limit of the 95 % confidence interval for the CI values lower than 0.9 (Fig. 2a). A similar pattern of this synergistic activity was also noted for the 3-ADON–15-ADON combination (Fig. 2c). In order to quantify the magnitude of the ascertained synergistic interactions, dose reduction indices (DRI) were calculated for low (10 %), medium (50 %) and high (80 %) cytotoxicity levels (Table 2). This later parameter indicates the ratio between the concentration of tested mycotoxins when used alone or in combination to achieve the same toxicity level. The DRI values for synergistic associations DON–15-ADON and 3-DON–15-ADON varied from 7 at lower toxicity level to 2 at higher toxicity level, indicating that the synergy had a higher magnitude at the low toxicity levels. Conversely, DON–NIV mixture depicted a constant-magnitude synergy that was less intensive (Fig. 2d; Table 2).

Other types of interaction between type B trichothecenes

By contrast to the DON–NIV mixture, the combination of DON to the acetyl derivative of NIV (FX) led to an antagonistic interaction, while the combination of NIV and FX was constantly additive (Fig. 2e, f). The DON–3-ADON mixture presented a unique feature of variation of the type of interaction accordingly to the dose. Low doses were antagonistic, and medium doses behaved additively, while synergy was only noted for the combined effects of high mycotoxin concentrations (Fig. 2b). The short-range synergy noted for DON–3-ADON mixture had a similar magnitude compared to other synergistic mixtures of DON and acetyl derivatives (Table 2).

Discussion

Fusarium toxins are very common contaminants for feedstuffs and food (Streit et al. 2012). Analytical results related to mycotoxin contamination of cereal grain commodities, collected in the course of national monitoring programmes in Finland, Sweden, Norway and the Netherlands during a 20-year period, revealed that DON had the highest overall incidence of 46 % (Van Der Fels-Klerx et al. 2012). It is likely that dietary exposure to mycotoxin may involve several compounds at the same time. During the last years, the shift from single analyte methods to multi-target methods has permitted a better comprehension of the reality of mycotoxin co-contamination of food matrices. There is increasing evidence that co-contamination of food matrices is the rule, not the exception (Streit et al. 2013). DON is often present with other type B trichothecenes such as 3-ADON, 15-ADON, NIV and FX (Eckard et al. 2011; Schollenberger et al. 2012; De Boevre et al. 2013) but variable amounts and ratios between these toxins have been reported. For example in maize silage, mean and maximum amounts for DON were 1,356 and 2,990 μg/Kg versus 521.3 and 760 μg/Kg for NIV, which correspond to an average ratio DON:NIV 2.6:1 (Eckard et al. 2011). In maize rudimentary ears, a higher contamination by NIV compared to DON was observed, 27,209 and 18,071 μg/Kg for the mean amounts, which corresponds to a DON:NIV ratio 1:1.15 (Schollenberger et al. 2012). For DON, 3-ADON and 15-ADON, the mean amounts in belgian cereal-based food products were 42, 25 and 16 μg/Kg respectively, which correspond to a DON:3-ADON ratio 1.68:1 and a DON:15-ADON ratio 2.63:1 (De Boevre et al. 2013).

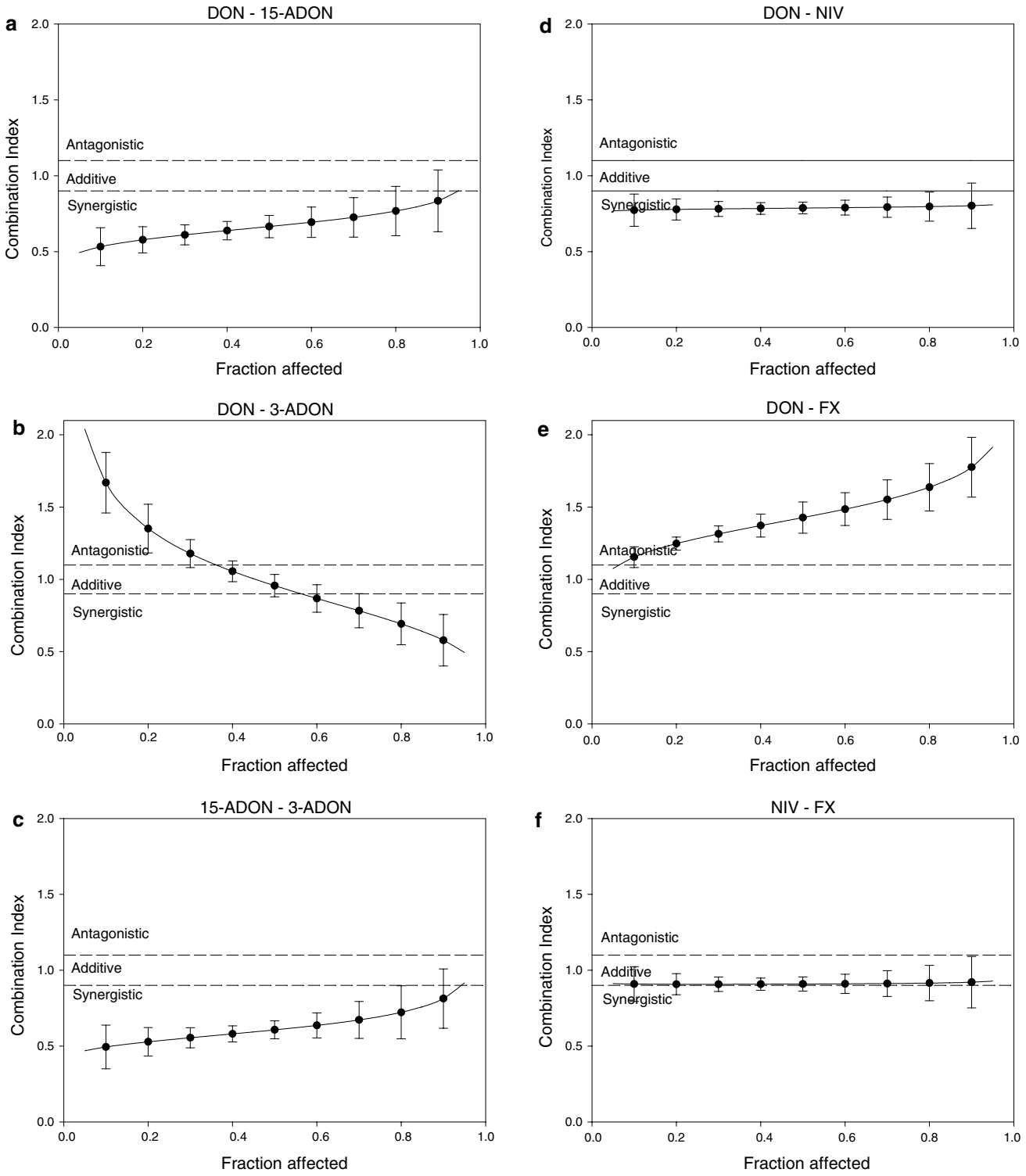

Fig. 2 Combination index—fraction affected curve for binary combinations of DON, NIV and their acetyl derivatives. CI values were calculated from data obtained from three independent experiments on the basis of roughly equipotent mycotoxin combinations. The *vertical bars* indicate 95 % confidence intervals for CI values based on sequential deletion analysis (Chou 2006). *Horizontal dashed lines* correspond to lower and upper limits of the additivity zone

Table 2 Combination Index and Dose reduction index values for viability inhibition by DON, NIV, their acetyl derivatives and their binary mixtures in IPEC-1 cells

Mycotoxin	Combination ratio	10 % Cytotoxicity		50 % Cytotoxicity		80 % Cytotoxicity	
		CI	DRI	CI	DRI	CI	DRI
DON	1:1	0.53	4.18	0.67	3.38	0.77	3.06
15-ADON			3.93		2.70		2.27
DON	1:10	1.67	2.07	0.96	2.63	0.69	3.06
3-ADON			0.84		1.73		2.73
15-ADON	1:10	0.49	6.50	0.61	3.65	0.72	2.53
3-ADON			2.94		3		3.05
DON	1:1	0.77	4.06	0.79	3.74	0.80	3.56
NIV			1.90		1.92		1.94
DON	1:0.8	1.15	1.67	1.43	1.45	1.64	1.33
FX			1.80		1.35		1.13
NIV	1:0.8	0.91	1.58	0.91	1.71	0.92	1.80
FX			3.64		3.09		2.79

CI: Combination Index. CI < 0.9, 0.9 < CI < 1.1, and CI > 1.1 indicate synergism, additive effect, and antagonism, respectively. DRI: Dose reduction index. DRI > 2 indicate a synergistic effect

The present study was designed to assess the combined toxicity of type B trichothecenes toward the proliferation of non-transformed intestinal epithelial cells.

Trichothecenes have multiple inhibitory effects on eukaryote cells, including inhibition of protein, DNA and RNA synthesis, inhibition of mitochondrial function, effects on cell division and membrane toxicity (Arunachalam and Doohan 2013). Therefore, organs and tissues showing high rates of cell turnover are regarded as particularly susceptible to trichothecenes. The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals (Heath 1996; Maresca et al. 2008). The high cell turnover allows rapid resealing of the epithelial barrier following injury or damage in order to maintain an effective barrier function. This normal homeostasis may be impaired by food contaminants affecting self-renewing capacities of the intestinal epithelium. Previous works showed that DON and its acetyl derivatives 3-ADON and 15-ADON alter pig intestinal barrier function by modulating the expression of tight junction proteins (Lucioli et al. 2013; Pinton et al. 2012). This work was performed to understand how DON, NIV and their acetyl derivatives could jointly impair intestinal barrier function by affecting epithelial cell renewing.

Pig is considered an illustrative case of dual-purpose target and model species that benefits agricultural and biomedical research, especially for the mycotoxin health risk assessment issues (Almond 1996; Ireland et al. 2008). The non-transformed porcine IPEC-1 cell line has been broadly used as an in vitro model for pig intestinal epithelium functionalities, to assess mycotoxins and especially fusariotoxins toxicity (Goossens et al. 2012; Loiseau et al. 2007; Pinton et al. 2010). This cell line is known to retain most of its original epithelial nature, which makes it possible to extrapolate the results of the in vitro trials to the in vivo situations (Brosnahan and Brown 2012; De Vos et al. 2012). Non-differentiated pig enterocytes appeared clearly more sensitive to DON toxicity (Vandenbroucke et al. 2011), suggesting that exposure to this contaminant could impair the physiologically constant state of regeneration of the intestinal epithelium. In this work, the mycotoxins and their mixtures clearly showed a dose-dependent toxicity toward proliferating IPEC-1 cells. The effective concentration inhibiting cell viability by 50 % (D_m) reported here for DON is in accordance with previous cytotoxicity studies using the IPEC-1 model (Danicke et al. 2010; Diesing et al. 2011). These results also confirm that 3-ADON exerts less toxicity to pig intestine than DON, while 15-ADON could be more toxic (Pinton et al. 2012). Compared to DON, NIV demonstrated a higher cytotoxic effect on IPEC-1 cells. A similar ranking was noted in the closely related IPEC J-2 cells, although the reported IC_{50} values were lower in a 48-h exposure study (Wan et al. 2013). Both cell lines are intestinal columnar epithelial cells derived from jejunum and a mixture of jejuna and ileal tissue respectively, from unsuckled 1-day-old piglets (Koh et al. 2008). In non-tumorigenic rat intestinal epithelial cell line IEC-6 also, NIV exerted a stronger anti-proliferative activity than DON (Bianco et al. 2012). The toxicity of NIV in this study was similar to the toxicity of FX (4-acetyl NIV) suggesting that the presence of an acetyl group may not increase the toxicity of NIV for IPEC-1 cells. Depending on the position of the acetyl radical on DON, the toxicity was lower (3-ADON) or higher (15-ADON). This is in line with the known structure–activity relationship of trichothecenes (Desjardins et al. 2007; Thompson and Wannemacher 1986). However the toxicity may vary depending on the biological systems. The toxicity ranking of NIV and its acetyl derivative FX in the mouse fibroblast 3T3 cell line was similar to the results presented here (Sundstol Eriksen et al. 2004). On the contrary a higher toxicity of FX

(10–30-fold increase) compared to NIV was noted in the human intestinal Caco-2 cell line (Alassane-Kpembi et al. 2013; Bony et al. 2007).

For interaction analysis, the critical issue is the definition of the additive response (also known as non-interaction response) and discrepancies in conclusion for interaction studies mainly result from the lack of a consensual definition of the additivity model. Most interaction studies consider that the additive response corresponds to the arithmetical summation of the individual effects of the compounds present in the mixture (Ficheux et al. 2012; Kouadio et al. 2007; Ribeiro et al. 2010). This approach is weakened by the hyperbolic or sigmoidal shape of most dose–response curves in toxicology (Boedeker and Backhaus 2010; Chou 2006). Indeed, doubling the dose does not automatically result in doubling the response. In the method used in the present study, the prediction of additive response is only based on a Mass-Action Law-based simulation of the combined effect (Chou 2006).

Using a factorial design approach, Wan et al. (2013) investigated the combined toxicity of *Fusarium* toxins DON and NIV on IPEC J-2, and concluded to a non-additive interaction. Unfortunately, this type of design did not allow specifying the type of interaction. The DON–NIV interaction was also studied on J7741.A, a murine macrophages cell line (Marzocco et al., 2009). By comparing the IC_{50} values for pro-apoptotic activity of the toxins and their mixture, the authors concluded to an additive interaction but the mathematical model behind the analysis was not clearly stated. In the present study the Chou-Talalay method was used to characterize the interactions induced by combinations of type B trichothecenes. This method combines a qualitative assessment of interactions via an isobolographic analysis and a quantification of synergy or antagonism by calculating a combination index and dose reduction index at different effect levels. The combined toxicity for DON and NIV could be characterized as a constant-magnitude synergy in IPEC-1 cells. Synergistic interaction between DON and NIV has already been reported in a yeast bioassay (*Kluyveromyces marxianus*). Indeed, the mixture demonstrated a higher degree of toxicity than that produced by the sum of the individual toxicities (Madhyastha et al. 1994). However because of the additivity model used in the study, the conclusion need confirmation. We also previously reported synergy combining DON and NIV on the one hand, and DON and its acetyl derivatives in human intestinal Caco-2 cells, even though the magnitude of interactions was higher at low doses (Alassane-Kpembi et al. 2013). These observations in IPEC-1 cells confirm previous conclusion in the Caco-2 model that when type B trichothecenes are present simultaneously in food and feed, the main type of interaction observed is synergy.

The dose reduction index, that is a quantitative assessment based on the ratio of observed to predicted doses of trichothecenes in mixtures, gives an indication of correction factors that may take the observed synergies into account (Chou 2006). In the present experiments the calculated correction factors ranged from 2 to 7. As already mentioned in the Caco-2 model, combining NIV and its acetyl derivative FX led to an additive toxicity. However, antagonism was noted with low-dose associations for the binary mixture DON–3-ADON and for the binary mixture DON–FX. It is noteworthy that these two acetyl derivatives showed a markedly lower individual toxicity in IPEC-1 compared to Caco-2 cells, which suggests that their biological pathways could differ in both cell lines (Alassane-Kpembi et al. 2013; Bony et al. 2007).

Though mainly synergistic effects have been observed in the present study, antagonism was reported for DON–3-ADON and DON–FX combinations. Moreover, concentration-dependent variations were noted for the magnitude and/or the type of interaction. Biologically, the involvement of cellular active transport systems could be proposed to explain the differences in intrinsic toxicity of the type B trichothecenes and the pattern of toxicological interactions reported in this study. Trichothecenes are small size amphiphatic organic molecules that contain aromatic groups. These structural criteria are common denominators identified for the efflux transporter P-gp substrates (Schinkel and Jonker 2003). It has been shown that DON and NIV may passively diffuse into intestinal cells and then become substrates for both P-gp and MRP2 efflux transporters (Tep et al. 2007; Videmann et al. 2007). One could speculate that differences in affinity to transporters may contribute to a differential cellular accumulation and the subsequent intrinsic toxicity of the type B trichothecenes. This involvement of transporters in the trafficking of trichothecenes also suggests that the mycotoxins could compete for binding when co-occurring. NIV by its own presented a limited bioavailability (Hedman et al. 1997; Poapolathep et al. 2003, 2004). In the DON–NIV mixture experiment, we could speculate that, competition for binding to transporters may have resulted in a higher accumulation of NIV depicted by a potentiation of toxicity for the mixture. The antagonism observed for DON–3-ADON and DON–FX mixtures may tentatively be explained by a higher affinity of the most toxic compound in each of the mixtures to the transporters, which could result in accumulation of the less toxic one and an overall lower toxicity than that would have been predicted for the combined effect. Finally, the variation of the magnitude and/or type of the interactions with the concentration may be explained by the graded levels of saturation of efflux systems from low to high toxin concentrations. These hypotheses deserve to be further investigated.

The present study demonstrates that the toxicity of a mixture of mycotoxins cannot be predicted solely on the basis of the effect of the individual compounds. The synergistic effects observed here after the exposure of a non-transformed cell line to the mixtures are biologically relevant since type B trichothecenes often occur in combination. The molecular mechanisms behind the toxicological interactions were not explored in this study and deserve further investigations. Besides these in vitro results further research into the toxicity of mycotoxin mixtures and their interactions in whole-animal assays should be addressed to provide regulatory agencies with substantial data in health risk assessment for the actual co-exposure to mycotoxins.

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Conflict of interest Authors declare no conflict of interest.

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