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New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic

Imourana Alassane-Kpembi [a,b,c], Martine Kolf-Clauw [a,b], Thierry Gauthier [a,b], Roberta Abrami [a,b], François A. Abiola [c], Isabelle P. Oswald [a,b,*], Olivier Puel [a,b]

[a] INRA, UMR 1331 Toxalim, Research Center in Food Toxicology, F-31027 Toulouse, France
[b] Université de Toulouse, ENVT, INP, UMR 1331 Toxalim, F-31076 Toulouse, France
[c] Institut des Sciences Biomédicales Appliquées, Cotonou, Bénin

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ABSTRACT

Deoxynivalenol (DON) is the most prevalent trichothecene mycotoxin in crops in Europe and North America. DON is often present with other type B trichothecenes such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon-X (FX). Although the cytotoxicity of individual mycotoxins has been widely studied, data on the toxicity of mycotoxin mixtures are limited. The aim of this study was to assess interactions caused by co-exposure to Type B trichothecenes on intestinal epithelial cells. Proliferating Caco-2 cells were exposed to increasing doses of Type B trichothecenes, alone or in binary or ternary mixtures. The MTT test and neutral red uptake, respectively linked to mitochondrial and lysosomal functions, were used to measure intestinal epithelial cytotoxicity. The five tested mycotoxins had a dose-dependent effect on proliferating enterocytes and could be classified in increasing order of toxicity: 3-ADON < 15-ADON ≈ DON < NIV ≪ FX. Binary or ternary mixtures also showed a dose-dependent effect. At low concentrations (cytotoxic effect between 10 and 30–40%), mycotoxin combinations were synergistic; however DON–NIV–FX mixture showed antagonism. At higher concentrations (cytotoxic effect around 50%), the combinations had an additive or nearly additive effect. These results indicate that the simultaneous presence of low doses of mycotoxins in food commodities and diet may be more toxic than predicted from the mycotoxins alone. Considering the frequent co-occurrence of trichothecenes in the diet and the concentrations of toxins to which consumers are exposed, this synergy should be taken into account.

Introduction

Mycotoxins are biologically active secondary fungal metabolites found as contaminants in almost all agricultural commodities worldwide, and they pose a major risk for human and animal health (Wild and Gong, 2010). Among them, the Type B trichothecenes constitute a group of toxins with a keto group at carbon 8 of the parent epoxytrichothecene nucleus. These compounds are produced by *Fusarium graminearum* and *Fusarium culmorum*, the main causal agents of *Fusarium* head blight, an important disease of small grain cereals worldwide.

Considered as phytotoxins, the trichothecenes favor the development of the fungus on the plant, although they are not necessary for the formation of the primary symptoms of the disease (Boenisch and Schafer, 2011). This family of toxins includes, but is not limited to, five closely related congeners: deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), fusarenon X (FX; 4-acetylnivalenol) and nivalenol (NIV) (Fig. 1). Due to a high prevalence of *Fusarium* head blight, Type B trichothecenes are the most common contaminants of cereal grains in temperate regions of the word. A large scale data survey indicates that DON, 15-ADON, NIV, FX and 3-ADON are present in 57%, 20%, 16%, 10% and 8%, respectively of food samples collected in the European Union (SCOOP, 2003).

The adverse effects of trichothecenes include emesis, nausea, anorexia, growth retardation, neuroendocrine changes and immunosuppression (Pestka, 2010). In humans, there is a body of evidences suggesting that trichothecenes cause acute illness and are frequently associated with outbreaks of gastroenteritis (Pestka, 2010). At the molecular level, trichothecenes display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA and RNA synthesis (Rocha et al., 2005). This impairment leads to the alteration in cell proliferation in tissue with high rates of cell turnover such as intestinal epithelial cells. Thus intestinal epithelial cells are especially sensitive to trichothecenes and their exposure to these toxins may induce toxicity (De Walle et al., 2010; Pinton et al., 2010). The intestine is also the first barrier to food

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; CI, combination index; DON, deoxynivalenol; 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.

* Corresponding author at: INRA, UMR 1331 Toxalim, Research Center in Food Toxicology, 180 chemin de Tournefeuille, BP93173, 31027 Toulouse Cedex 03, France.
E-mail address: Isabelle.Oswald@toulouse.inra.fr (I.P. Oswald).

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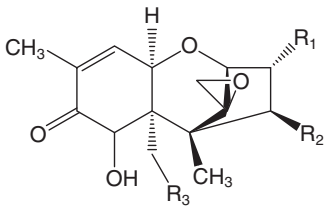

	R_1	R_2	R_3
Deoxynivalenol	OH	H	OH
Nivalenol	OH	OH	OH
Fusarenon X	OH	OAc	OH
15 Acetyl-deoxynivalenol	OH	H	OAc
3 Acetyl-deoxynivalenol	OAc	H	OH

Fig. 1. Chemical structures of DON and NIV and their acetyl derivatives.

contaminants. Following the ingestion of mycotoxin-contaminated food or feed, intestinal epithelial cells can be exposed to high concentrations of toxins (Maresca et al., 2008).

Human and animals are exposed simultaneously to several trichothecenes for at least three different reasons: (i) most *Fusarium* are able to produce a number of mycotoxins simultaneously, (ii) food commodities can be contaminated by several fungi simultaneously or in quick succession and (iii) a complete diet is made up of various different commodities. Humans may also be exposed to multiple trichothecenes via products from animals that have eaten contaminated feed (Streit et al., 2012). Rodrigues and Naehrer (2012) screened 7049 feed and feedstuff samples in a three-year survey on the worldwide occurrence of mycotoxins, and reported 48% to be contaminated by two or more mycotoxins. From a total of 29 wheat samples collected in three EU countries, Monbaliu et al. (2010) reported that 75% of the contaminated samples were positive for more than one type of mycotoxin. In the case of trichothecenes, several studies have shown a co-occurrence of trichothecenes in corn, wheat and barley (Eckard et al., 2011; Hajslova et al., 2007; Kim et al., 1993; Schollenberger et al., 2012).

Because of their natural co-occurrence, there is an 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 (CAST, 2003; Grenier and Oswald, 2011). Multi-exposure may lead to additive, synergistic or antagonist toxic effects. The data on toxic effects of mycotoxin mixtures are limited and therefore, the actual health risk from exposure to the combination of mycotoxins is unknown. Indeed, there are very few studies addressing the combined effects of mycotoxins and at present the database describing the possible effects of combined exposure of trichothecenes is very sparse and not sufficient to establish either the nature of combined effect or the relative potencies of these toxins.

The aim of the present study was to establish a relative potency scale for five Type B trichothecenes and to assess their combined effects in terms of additive, antagonistic or synergistic toxicity towards human intestinal epithelial cells.

Materials and methods

Toxins

DON, 3-ADON, 15-ADON were purchased from Sigma (St Quentin Fallavier, France); NIV, FX from Waco Pure Chemical Industries LTD (Osaka, Japan). Stock solutions of mycotoxins were dissolved in DMSO to the following concentrations: 15 mM DON and 15-ADON, 20 mM 3-ADON, 10 mM NIV and FX. Stock solutions were stored at −20 °C and working dilutions were prepared in cell culture medium. Final concentration of 0.1% DMSO corresponding to the highest DMSO concentration of working dilutions was tested and results were not significantly different from controls.

Cell culture and reagents

The Caco-2 cell line has been derived from a human colon adenocarcinoma (ATCC HTB-37, Rockville, MD, USA). For these experiments, Caco-2 cells were grown in 75-cm^2 culture flasks in culture medium consisting of Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum (Perbio Sciences, Bezons, France), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 UI/mL penicillin, and 100 μg/mL streptomycin (Eurobio, Courtaboeuf, France) in an atmosphere of 5% CO_2 at 37 °C. Before reaching confluence, the cells were trypsinized and plated in 96-well flat-bottom cell culture plates (Costar, Cambridge, MA, USA) for performing cytotoxicity assay.

Cytotoxicity assays

Two cytotoxicity assays, the MTT test and neutral red uptake, were performed to assess the individual and combined effects of DON, NIV and their acetyl derivatives. Proliferating Caco-2 cells (10 000 cells per well) were seeded in 96-well plates, and incubated for 24 h at 37 °C, before a 48-hour exposure to mycotoxins alone or in mixtures. Negative controls were obtained by the treating cells with the solvent alone (DMSO). The final toxin concentrations tested ranged from 7.5 nM to 6.67 μM. The tested binary combination ratios were: 1/1 for DON/15-ADON, 1/1.67 for DON/3-ADON, 1/0.8 for DON/NIV, 1/0.03 for DON/FX and 1/0.04 for NIV/FX. The tested ternary ratios were: 1/1/1.67 for DON/15-ADON/3-ADON and 1/0.8/0.03 for DON/NIV/FX. These ratios, calculated from preliminary individual cytotoxicity experiments, enabled a similar toxicity to be obtained for each mycotoxin.

The MTT test was performed as described by Gauthier et al. (2012). The neutral red uptake cytotoxicity assay was run according to Repetto et al. (2008).

For both tests, the percentage of viable cells was calculated using the formula:

$$\text{Viability}\,(\%) = 100 \times \frac{\text{Mean OD of mycotoxin(s) treated sample}}{\text{Mean OD of untreated sample}}.$$

Data analysis

Individual mycotoxin cytotoxicity. The dose–response relationships of the individual 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 effects plots were greater or equal to 0.95 (Chou, 2011).

Effects of mixtures. Dose–response curves for single mycotoxins, and binary or ternary associations were generated simultaneously. Mycotoxin interactions were analyzed by the isobologram and combination-index methods derived from the median effect principle of Chou and Talalay (1984).

Isobolograms were drawn for a binary combination of mycotoxins at doses inducing 10, 30 and 50% cytotoxicity as previously described elsewhere (Kolf-Clauw et al., in press). The combination-index (CI)

method was also used to analyze the mycotoxin interaction. This index is calculated according to Chou (2011):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

where $^{n}(CI)_x$ is the combination index for n toxins at x% inhibition, $(D)_j$ is the doses of n toxins that exerts x% inhibition in combination, $(D_x)_j$ is the doses of each of n toxins alone that exerts x% inhibition. CI = 0.9 − 1, CI < 0.9, and CI > 1.1 indicate an additive effect, a synergism, and an antagonism, respectively, regardless of the mechanisms or the units of the drugs.

For all binary and ternary mycotoxin combinations, CI values were generated over a range of fractions of cell viability affected (f_a) from 0.05 to 0.95 (5% to 95% toxicity).

When synergy occurred in the effects of the mixtures, dose reduction indices (DRI) were calculated. The dose reduction index 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. The dose reduction index for each drug can easily be obtained by setting the reciprocal of the CI equation (Chou, 2011):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{1}{(DRI)_j}$$

and

$$(DRI)_1 = \frac{(D_x)_1}{(D)_1}, \quad (DRI)_2 = \frac{(D_x)_2}{(D)_2} \ldots, \text{etc.}$$

Dose–effect curves analysis. The dose–response relationship analysis for individual mycotoxin cytotoxicity, CI and their 95% confidence interval 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).

Statistical analysis. The reported values are the means ± standard deviation (SD) of at least three independent experiments, each with triplicate wells per dose level. Statistical analyses were performed using SigmaPlot 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.

Results

Comparative cytotoxicity of Type B trichothecenes on Caco-2 cells

Two cytotoxicity assays, based respectively on mitochondrial and lysosomal activities, were used to compare the toxic effect of Type B trichothecenes. Proliferating Caco-2 cells were exposed for 48 h to concentrations of mycotoxins ranging from 7.5 nM to 6.67 μM. The five mycotoxins had a dose-dependent effect on both neutral red uptake and MTT activity (Fig. 2) with mean IC_{50} values ranging from 20 nM to 3 μM (Table 1).

For the overall ranking, mycotoxins were cytotoxic for proliferating enterocytes in the increasing order 3-ADON < 15-ADON ≈ DON < NIV ≪ FX.

Combined cytotoxicity of Type B trichothecenes

The next step was to determine the cytotoxicity of Type B trichothecene mixtures and to characterize the type of interaction between these mycotoxins when they are present together (additivity, synergy or antagonism). The combination ratios, calculated on the basis of the IC_{50} values obtained in individual cytotoxicity experiments, were chosen to obtain an equipotent toxicity for each mycotoxin in a mixture. The cytotoxicity was assessed on proliferating Caco-2 cells using neutral red test, as this assay was more sensitive than the MTT test (Table 1). Both single mycotoxins and binary or ternary mixtures showed a dose-dependent effect (Figs. 3 and 4). Isobolograms were drawn and CI values were calculated for different toxicity levels. When a synergistic effect was observed, the dose reduction indices were calculated to quantify the synergy.

Combined toxicity of DON, 15-ADON and 3-ADON. DON, 15-ADON and 3-ADON, and their binary and ternary combinations, caused a dose-dependent toxicity in Caco-2 cells (Fig. 3). When DON was associated with its acetylated derivatives, an additive effect was observed at the 50% growth inhibition level, while a synergistic effect was noted at lower cytotoxic levels (Figs. 5 and 6).

The Fig. 5 presents the isobologram for the combinations of DON and 3-ADON at three different levels of inhibition (10%, 30% and 50%). In this type of graph, the additive 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 antagonist or a synergistic effect of the combination respectively. In this figure, we can observe the additive effect of DON and 3-ADON at the 50% cytotoxicity level and the synergistic effect at lower cytotoxicity levels. The interaction between DON and its acetylated derivates was further analyzed by calculating CI value at various cytotoxicity levels. The CI/f_a curves for binary and ternary

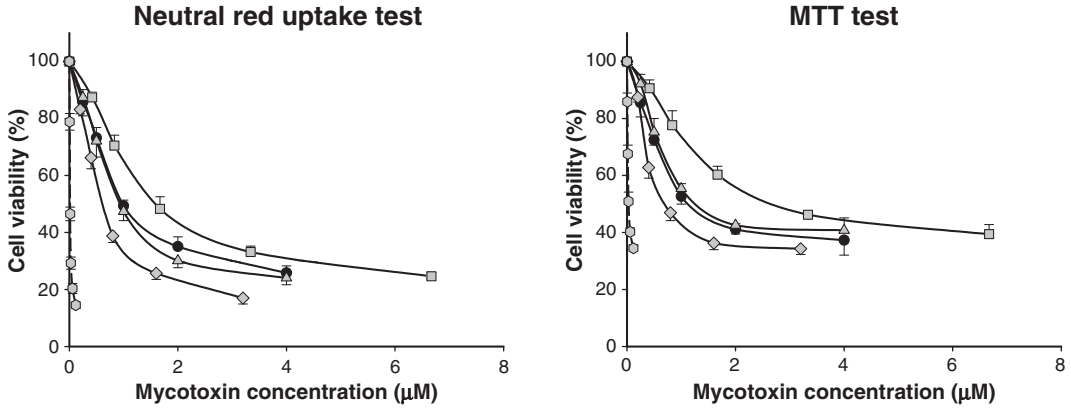

Fig. 2. Comparative cytotoxicity of Type B trichothecenes on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of DON (○), 3-ADON (□), 15-ADON (△), NIV (◇) or FX (⬭). Cytotoxicity was assessed by the MTT and neutral red assays. Data are means ± SD of three to four independent experiments.

Table 1
Comparison of the cytotoxicity of Type B trichothecenes against Caco-2 cells after a 48 h exposure using the MTT and neutral red tests.

Mycotoxin	IC_{50} (μM)	
	MTT	Neutral red
DON	1.39 ± 0.07^{a}	1.19 ± 0.29^{a}
3-ADON	2.94 ± 0.45^{ab}	1.99 ± 0.44^{b}
15-ADON	1.47 ± 0.28^{abc}	1.1 ± 0.38^{ac}
NIV	0.9 ± 0.24^{cd}	0.69 ± 0.16^{cd}
FX	0.04 ± 0.01^{e}	0.02 ± 0.00^{e}

Data are means ± SD of three to four independent experiments.
^{abcde}Means in a column without a common letter differ ($p < 0.05$).

combinations of DON, 3-ADON and 15-ADON are shown in Fig. 6. At low concentrations (f_a between 10% and 30–40%), mycotoxin combinations give a CI < 1 indicating a synergistic toxic effect. At higher concentrations (f_a between 30–40 and 50%), mycotoxin combinations have a CI around 1, indicating an additive effect. Conversely antagonism was observed at 70% cytotoxicity and above (data not shown).

In order to quantify the synergy between DON and its acetyl derivatives, dose reduction indices were calculated for 10% and 30% level of cytotoxicity (Table 2). This latter parameter indicates the ratio between the concentration of tested mycotoxins when used alone or in combination to achieve the same toxicity level. For the ternary mixture DON/15-ADON/3-ADON, the dose reduction indices of the three individual mycotoxins ranged from 6.8 to 10 at 10% toxicity and from 3.4 to 5 at 30% toxicity. For binary mixtures, the dose reduction indices range from 2.8 to 4.3 at 10% toxicity and 2 to 3.2 at 30% toxicity.

Combined toxicity of DON, NIV and FX. The combined toxicity of DON, NIV and FX was also studied on proliferating Caco-2 cells (Figs. 4 and 7). As already observed with DON and acetylated derivatives, when DON was associated with NIV or FX, a synergy was observed at low cytotoxicity levels (Fig. 7). Above 50% toxicity the interaction turned into an additive effect (data not shown). The dose reduction index values were higher at 10% toxicity than at 30% toxicity (Table 2). Compared to single compounds, the toxicity of the DON-FX combination showed a 6-fold concentration reduction for DON and a 3-fold reduction for FX. DON and NIV concentrations showed a 3-fold reduction in a binary mixture. Whatever the toxicity level, the NIV–FX combination gave an additive interaction.

When the three toxins were present together, an antagonism was observed at 10 and 20% of toxicity. The calculated dose reduction index values were 3, 3 and 1.6 respectively for DON, NIV and FX indicating a weaker effect of FX in the mixture compared to that predicted on the basis of additivity.

Discussion

DON is the most prevalent trichothecene mycotoxin present in crops in Europe and North America (CAST, 2003; SCOOP, 2003). Consumers are thus particularly concerned over the exposure to this toxin as indicated by recent survey (Turner et al., 2008). The second French total diet study highlights that exposure to DON significantly exceeds the health-based guidance values (Sirot et al., 2013). DON is often present with other Type B trichothecenes such as 3-ADON, 15-ADON, NIV and FX (Schollenberger et al., 2012). The aim of this

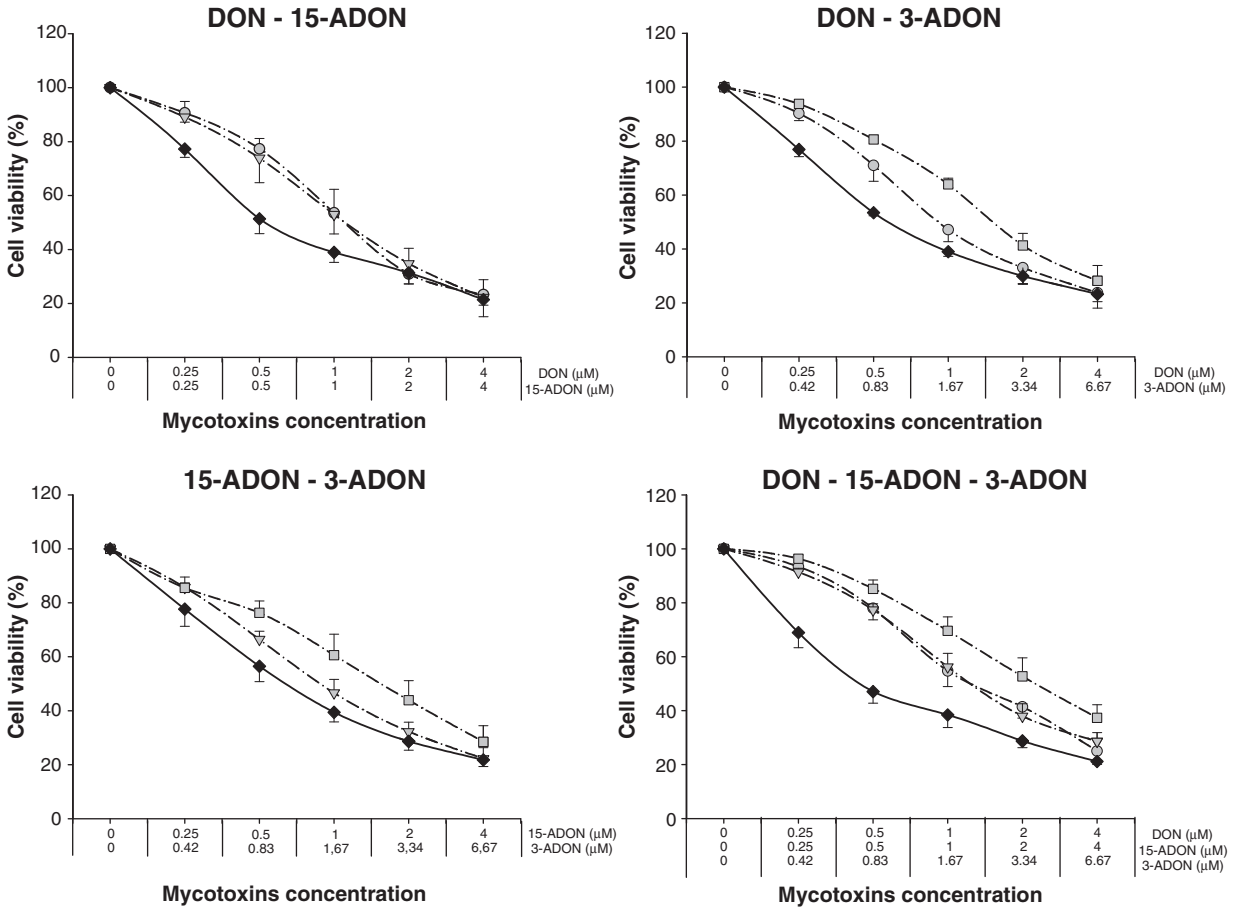

Fig. 3. Comparative toxicity of DON (○), 3-ADON (□) and 15-ADON (▽) alone or in binary or ternary mixtures/combinations (♦) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral red assay. Data are means ± SD of three independent experiments.

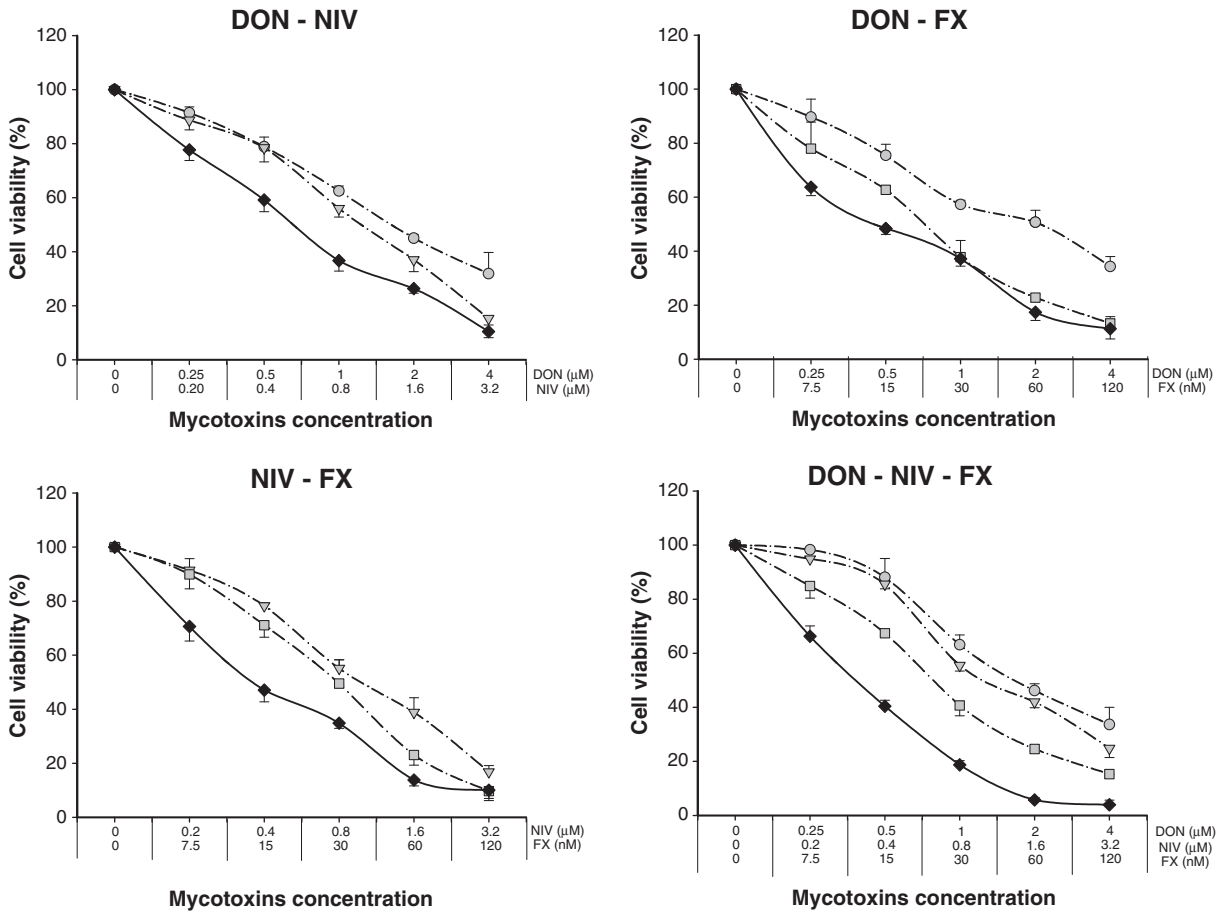

Fig. 4. Comparative toxicity of DON (○), NIV (▽) and FX (□) alone or in binary or ternary mixtures/combinations (♦) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral red assay. Data are means ± SD of three independent experiments.

study was to assess the interactions that occur when there is co-exposure to Type B trichothecenes.

These mycotoxins have been shown to alter several functions of intestinal epithelial cells including cell proliferation, barrier function, nutrient absorption and immune responses (Bianco et al., 2012; Diesing et al., 2012; Maresca et al., 2002). The MTT test and neutral red uptake, respectively linked to mitochondrial and lysosomal functions, were used for the indirect measurement of proliferation and viability of Caco-2 cells. By these two tests, we demonstrated that Type B trichothecenes, alone or in combination, were cytotoxic for intestinal cells in a dose-dependent manner. The IC_{50} and the relative toxicity of the five selected mycotoxins were similar with both cytotoxicity tests.

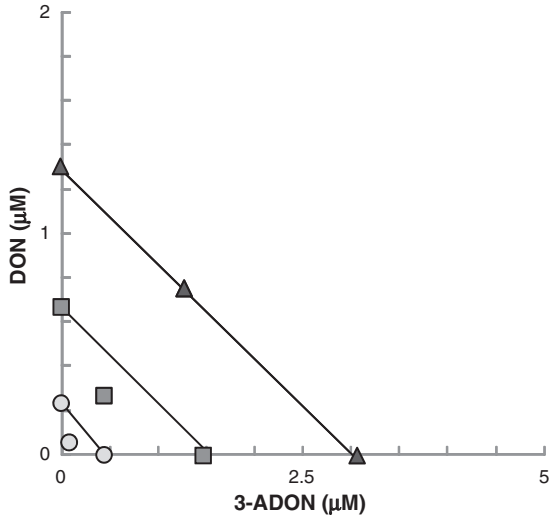

Fig. 5. Isobologram illustrating the combined cytotoxicity of DON and 3-ADON. At concentrations eliciting 10% (○), 30% (■) or 50% (▲) toxicity. The points are mean concentrations of dose–response neutral red cytotoxicity curves for each toxin or toxin combination (Compusyn software analysis).

In this study, we observed that NIV was 20–35 times less toxic to Caco-2 cells than its acetyl derivative FX. A slightly less relative potency (10–20) was reported in the same cell line by Bony et al. (2007). In other cell lines, FX has been found to be more toxic than NIV in unsettled proportions (Forsell and Pestka, 1985; Sundstol Eriksen et al., 2004; Thompson and Wannemacher, 1986). The IC_{50} values calculated in the current study for NIV and DON are in accordance with those obtained by Nielsen et al. (2009) in Caco-2 and other human cell lines, confirming the higher toxicity of NIV compared to DON. By MTT and neutral red uptake assays, 3-ADON was 2-fold less toxic for proliferating Caco-2 cells than DON and 15-ADON. This result confirms the lower toxicity of 3-ADON observed in other previous studies (Daenicke et al., 2011; Pinton et al., 2012; Sundstol Eriksen et al., 2004; Thompson and Wannemacher, 1986; Visconti et al., 1991) even though the difference in toxicity between 3-ADON and the two other trichothecenes varied significantly from one study to another.

Binary and ternary combinations of Type B trichothecenes were tested for interaction using the Chou-Talalay method (Chou, 2006). This method, already used to study other mycotoxin combinations (Jones et al., 1995; Koshinsky and Khachatourians, 1992; Ruiz et al.,

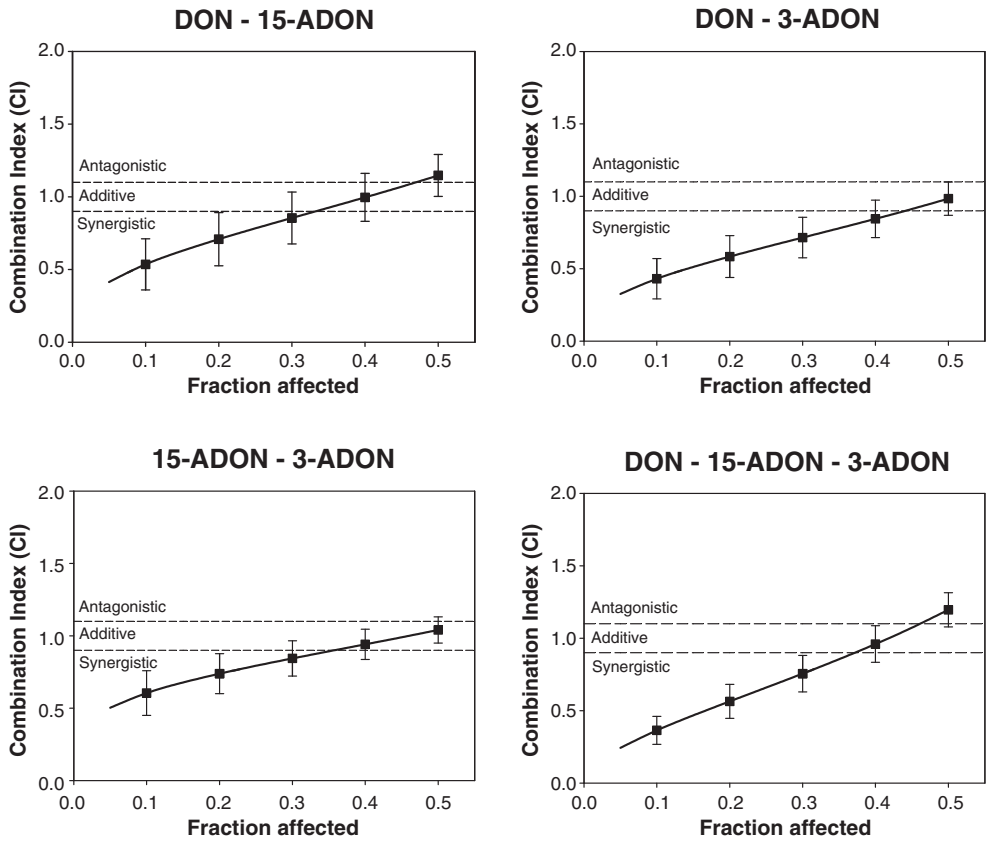

Fig. 6. Combination index — fraction affected curve for binary and ternary combinations of DON, 3-ADON and 15-ADON. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (Chou, 2006). Horizontal dashed lines correspond to lower and upper limits of the additivity zone.

2011) 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. For the 50% cytotoxicity level, the isobolographic analysis indicates an additive effect of the toxins. This pattern was confirmed by the combination index, calculated for the binary and ternary mixtures. Additive interaction between DON and NIV has already been reported in several cell types such as murine J7741A macrophages (Marzocco et al., 2009), porcine whole blood cells (Luongo et al., 2008), and human lymphocytes (Thuvander et al., 1999). To the best of our knowledge, ours is the first study that assessed globally the combined effect of Type B trichothecenes.

Table 2
Low-dose synergy of Type B trichothecenes for intestinal cytotoxicity.

Mycotoxin	Combination ratio	10% cytotoxicity		30% cytotoxicity	
		CI	DRI	CI	DRI
DON	1:1	0.54	4.2	0.85	2.5
15-ADON			3.4		2.2
DON	1:1.67	0.43	4.3	0.71	2.5
3-ADON			5		3.2
15-ADON	1:1.67	0.61	2.8	0.84	2
3-ADON			4		2.9
DON	1:1.67:1	0.37	8.5	0.76	3.8
3-ADON			10		3.4
15-ADON			6.8		5
DON	1:0.8	0.73	2.8	0.81	2.7
NIV			2.7		2.2
DON	1:0.03	0.48	6	0.66	4.7
FX			3.2		2.3
NIV	1:0.04	0.82	3.5	0.9	3
FX			1.9		1.8
DON	1:0.8:0.03	1.33	–	1.07	–
NIV			–		–
FX			–		–

Dose reduction indices were calculated by comparing the concentration required to reach 10 and 30% cytotoxicity when the mycotoxin was used singly and in combination. DRI > 2 for binary combinations and DRI > 3 for ternary combinations indicate a synergistic effect.

The main results of this paper are the observations that (i) the type of interaction varies with the cytotoxicity level and (ii) below a cytotoxicity level of 50%, the combined effects of binary or ternary mixtures of Type B trichothecenes are synergistic. In the present study a synergistic effect of DON, when combined with other Type B trichothecenes, was observed at IC_{10} and IC_{30}, i.e. at 0.15 to 0.55 μM DON. These findings suggest that the simultaneous presence of low doses of mycotoxins in food commodities and diet may induce greater toxicity than that can be predicted from the mycotoxins alone. This observation is of high biological relevance if we consider the concentrations of mycotoxins to which consumers are exposed. Indeed, DON concentrations of 0.16–2 μg/mL (0.5–7 μM) can be considered as realistic in human gut (Sergent et al., 2006). The lower concentration value corresponds to the mean estimated daily intake of French adult consumers on a chronic basis (Sirot et al., 2013). The higher concentration value simulates level that can be reached after the consumption of heavily contaminated food, as can be occasionally encountered. The dose reduction index, that is a quantitative assessment based on the ratio of observed to predicted doses of trichothecenes in mixtures, permitted the calculation of correction factors that may take the observed low-dose synergies into account. In the present experiments the calculated correction factors ranged from 2 to 10. To the best of our knowledge, this is the first study that mentions the magnitude of mycotoxin synergy. A lack of quantitative estimates of the magnitude of interactions has been pointed as a weakness of chemical combined effect

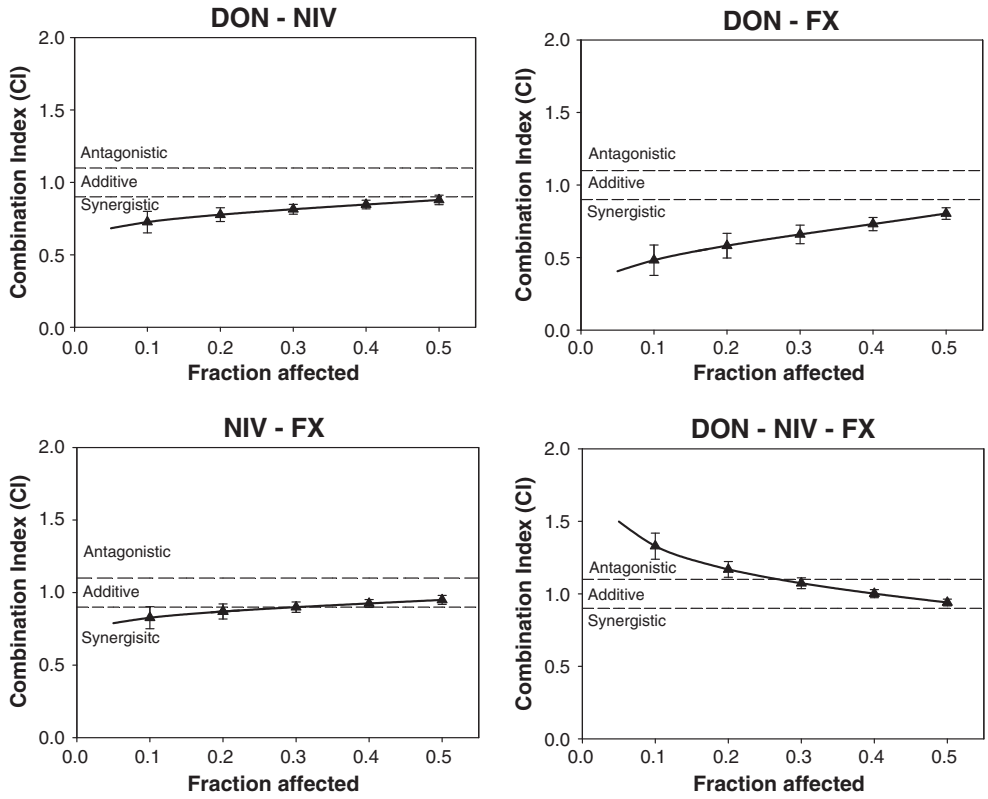

Fig. 7. Combination index — fraction affected curve for binary and ternary combinations of DON, NIV and FX. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (Chou, 2006). Horizontal dashed lines correspond to lower and upper limits of the additivity zone.

studies. In a critical analysis of 90 papers on mixture toxicity, Boobis et al. (2011) identified only 11 papers that mentioned the interaction magnitude.

The ternary combination of DON–NIV–FX showed antagonism for 10% cytotoxicity. This later antagonistic interaction seems to be linked to a lower toxicity of FX in the mixture as shown by the lower dose reduction index value. The higher toxicity of FX is mainly due to its higher hydrophobicity that facilitates its passage across the apical membrane and a de-acetylation step leading to NIV accumulation in the enterocytes (Ohta et al., 1978; Poapolathep et al., 2003). The reduction of FX toxicity might be due to the competition of DON and NIV at the level of substrate binding sites of the de-acetylase leading to a reduced de-acetylation of FX. This hypothesis deserves to be further investigated.

The Caco-2 cell represents a well-established model for the study of intestinal transport, metabolism and toxicity of nutrients and xenobiotics, and is wildly used in pharmacology and toxicology (Artursson et al., 2001; Boveri et al., 2004). However a number of limitations reported for this model, especially in drug discovery and mechanistic studies, arise critical issues in extrapolating the in vitro results to in vivo situations (Press and Di Grandi, 2008; Sun et al., 2002). In term of cytotoxicity, similar IC_{50} values for DON and NIV were reported for Caco-2 and seven other human permanent cell lines (Nielsen et al., 2009). Nevertheless, the data obtained in this paper, especially the synergy observed at low mycotoxin concentrations should be confirmed using in vivo or ex vivo models (Grenier and Oswald, 2011; Kolf-Clauw et al., 2009).

The present study demonstrates that the effect of a mixture of mycotoxins cannot be predicted solely on the basis of the effect of the individual compounds. Our results clearly indicate that the susceptibility of Caco-2 cells to the mycotoxins differed between the combinations assayed. The mechanism(s) of these interactions deserves further investigations. The synergistic effects observed after cell exposure to a mixture of low concentrations of mycotoxins tested are of practical importance since trichothecenes often occur in combination. The synergistic effect observed could pose a significant threat to public health (Speijers and Speijers, 2004). Considering the co-occurrence of mycotoxins in food, further research into the cytotoxicity of mycotoxin mixtures and their interactions should be addressed. Moreover, given the mycotoxin interactions, government regulatory standards about a great variety of mycotoxins or their mixtures are needed. New risk assessment strategies should take into account the toxicological interactions of mycotoxins in food and feed as already suggested (SCF, 2002).

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Conflict of interest

Authors declare no conflict of interest.

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