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Co-exposure to low doses of the food contaminants Deoxynivalenol and Nivalenol has a synergistic inflammatory effect on intestinal explants

Imourana Alassane-Kpembi[a,b], Olivier Puel[a], Philippe Pinton[a], Anne–Marie Cossalter[a], Ting-Chao Chou[c,d] and Isabelle P. Oswald[a]
[a]Toxalim, Research center in Food Toxicology, Université de Toulouse, INRA, ENVT, INP- PURPAN, UPS, F-31027, Toulouse France
[b]Hôpital d'Instruction des Armées, Camp Guézo 01BP517 Cotonou, Bénin
[c]Molecular Pharmacology & Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
[d]Present address: PD Science LLC, Paramus, NJ 07652-1754 USA

Corresponding author: Isabelle P. OSWALD, INRA, UMR 1331 Toxalim, Research Center in Food Toxicology, 180 Chemin de Tournefeuille, BP93173, 31027 Toulouse cedex 03, FRANCE. Isabelle.Oswald@toulouse.inra.fr, Tel: 33 (0) 5 61 28 54 80

Running title: Synergistic inflammatory effect of trichothecenes

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Abstract

The global incidence of Fusarium head blight and attendant cereal grains multi-contamination by the trichothecene mycotoxins deoxynivalenol (DON) and nivalenol (NIV) are increasing as a possible result of climate change and inadequate agricultural practices. At the molecular level, these mycotoxins bind to the ribosome, activate the mitogen-activated protein kinase and induce a local and systemic inflammation. DON is of public health concern owing to the narrow margin between exposure and tolerable daily intake. The intestinal inflammatory response to DON, NIV and their mixture was analyzed to determine thresholds for their intestinal pro-inflammatory effects and characterize the type and magnitude of their interaction. Fully differentiated-three dimensional porcine jejunal explants were exposed to increasing doses of DON and NIV alone or in combination; the expression level of IL-1α, IL-1β, IL-8, IL-17A, and IL-22 was measured by RT-PCR. Doses as low as 0.16µM DON or 0.73µM NIV significantly increase the intestinal expression levels of the tested inflammation-related genes. These doses are lower than those previously reported for other intestinal toxicity endpoints. The combined pro-inflammatory activity of DON and NIV was synergistic for all the tested genes with combination-index value range of 0.23 – 0.8. Our results indicate that (i) inflammation is a very sensitive endpoint for the intestinal toxicity of the trichothecenes, and (ii) co-exposure to DON and NIV has a greater inflammatory effect than induced by mycotoxins alone. This synergy should be taken into account considering the frequent co-occurrence of DON and NIV in the diet.

Keywords: Low-dose synergy; deoxynivalenol; nivalenol; pro-inflammatory cytokine; intestine; Benchmark Dose

1. Introduction

Type B trichothecenes are a group of mycotoxins that mainly contaminate cereals following fungal infection by *Fusarium* species (namely *F. graminearum* and *F. culmorum*) in the field and/or as part of postharvest spoilage. This group of toxins includes deoxynivalenol (DON) and nivalenol (NIV) and their acetylated precursors. DON is regarded as an important food safety issue since it is the most prevalent mycotoxin in Europe and North America (CAST 2003; EFSA 2013a; Schothorst and van Egmond 2004). Moreover, exposure assessments conducted in European countries concluded that high consumers and young children are exposed to DON at levels close to, or even higher than the Tolerable Daily Intake (TDI) (EFSA 2013a; Sirot et al. 2013). A number of studies have stressed the co-occurrence of *Fusarium* mycotoxins, and the simultaneous exposure to both DON and NIV is attested by several bio-monitoring reports as well as commercial and field products surveys (Abia et al. 2013; Del Ponte et al. 2012; Fredlund et al. 2013; Tanaka et al. 2010). Unlike DON, the consumers are not considered at risk for their sole exposure to NIV, since their daily intake remains below the TDI (EFSA 2013b) (EFSA 2013b). However, the implications of the combined effects of DON, which exposure is close and sometimes higher than the TDI, and NIV, have not received adequate attention. Furthermore, there is converging evidence that some mycotoxins, especially trichothecenes, could synergistically interact for toxicity, heightening concerns about health risks (Alassane-Kpembi et al. 2013; Alassane-Kpembi et al. 2016; Tajima et al. 2002).

The adverse effects of DON and NIV primarily include emesis, nausea, anorexia, growth retardation, neuroendocrine changes and immunomodulation (Pestka 2010a). At the molecular level, the capacity of trichothecenes to inhibit protein synthesis upon their binding to ribosomal RNA is pivotal for their effects (Pierron et al. 2016a). This binding to ribosome rapidly activates mitogen-activated kinases (MAPKs) via a so-called "ribotoxic stress" response (Iordanov et al. 1997; Zhou et al. 2003). The

DON-mediated activation of ERK and p38 MAPKs induces trans-activation of a number of pro-inflammatory cytokines and increases the stability of their mRNA (Pestka 2010b).

The newly described Th17 cells seem to be prominently involved in the gut inflammatory response of both Crohn's disease and ulcerative colitis (Scaldaferri and Fiocchi 2007). "Our lab previously reported that, by contrast to the regulatory Th17 (rTh17)-related genes, intestinal exposure to DON induces a profound alteration of the expression of IL-17A, IL-22 and IL-23A genes related to the pathogenic Th-17 inflammatory response resulting in the up-regulation of IL-17A, IL-22 and IL-23A cytokines (Cano et al. 2013)".. Based on the ability of DON to cause intestinal immune alterations mimicking the one found in inflammatory bowel diseases, it is proposed that DON may play a role in such diseases (Cano et al. 2013; Maresca 2013; Payros et al. 2016). In contrast to DON, little is known about the pro-inflammatory activity of NIV, especially at the intestinal level.

In this study we investigated the intestinal inflammatory response following exposure to DON, NIV and their mixture.

2. Material and methods

2.1 Chemicals

Deoxynivalenol (DON) purchased from Sigma (St Quentin Fallavier, France) and nivalenol (NIV) purchased from Waco Pure Chemical Industries LTD (Osaka, Japan) were dissolved to 60 mM in dimethylsulfoxide (DMSO), and stored at −20°C before dilution in complete culture media. Control samples were treated with equivalent concentrations of DMSO, which were verified to be non toxic for intestinal explants (data not shown).

2.2 Explants cultures

Jejunal tissue was obtained from six piglets 7–days after weaning (5 week-old at the time of the experiment). Animals were fed *ad libitum* prior to the experiment. The animal experiment was carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes.

Explants were prepared as already described (Lucioli et al. 2013). Briefly, a 5 cm middle jejunum segment was collected in pre-warmed Williams media (Sigma) supplemented with 200U/mL penicillin and 200 µg/mL streptomycin (Eurobio, Courtaboeuf, France). It was washed twice and opened longitudinally. Then, the external *tunica muscularis* was removed and explants were made with punch trocars (Centravet, Lapalisse, France) and were placed in Williams culture media supplemented with 1% of penicillin/streptomycin, 0.5% of gentamycine (Eurobio), 4.5 g/L of glucose (Sigma), 10% FBS (Eurobio) and 30 mM of amino acid (Ala/Glu) (Eurobio). Explants were exposed to serial dilutions of purified DON or NIV or their equimolar mixture (ratio 1:1) at concentrations ranging from 0 to 40 µM and incubated at 39°C in a humidified atmosphere with 5% CO_2 for 5 hours. After treatment, tissues were snap-frozen and stored at -80°C for transcriptional analysis.

2.3 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR (qPCR) was performed to determine the relative mRNA expression levels of the chemokine IL-8 and the pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-17A and IL-22. RNA concentration, integrity and quality were determined using Nanodrop ND1000 (Labtech International, Paris, France) and analysis of the electrophoregram generated by an Agilent Bioanalyzer (Agilent RNA 6000 Nano Kit Quick, Agilent Bioanalyzer 2100). RNA Integrity Numbers (RINs) of these mRNA were around 6.10. Then reverse transcription and real-time qPCR steps were performed as previously described (Grenier et al. 2011; Halloy et al. 2005). Non-reverse transcripted RNA was used as non-template control for verification of genomic DNA amplification signal. Specificity of qPCR products was assessed at the end of the reactions by analyzing dissociation curves. Primers were purchased from Invitrogen (Invitrogen, Life Technologies Corporation, Paisley, UK). Specific sequences are indicated in Table 1. Ribosomal protein L32 (RPL32) and β2-Microglobulin were used as housekeeping genes. For quantification and statistical analysis, target genes mRNA expression was normalized to the expressed housekeeping genes using REST© 2009 software (Qiagen, Valencia, CA) which uses the pair-wise fixed reallocation randomization test as statistical model.

2.4 Benchmark dose analysis of PODs for the pro-inflammatory effects of DON and NIV

A Benchmark dose (BMD) analysis was conducted for deriving the points of departure (PODs) for the pro-inflammatory effects of DON and NIV (Crump 1995). BMD is defined as the dose corresponding to a predetermined change in adverse response, the benchmark response (BMR), compared to the response in the control group. As suggested by U.S.EPA (1995), one standard deviation above the control mean was used to set the BMR. PROAST (Possible Risk Obtained from Animal Studies) software was used for calculation of the BMDs and their lower confidence bounds (BMDLs) (http://www.rivm.nl/proast). These BMDLs were considered as the points of departure for the pro-inflammatory gene activation by DON and NIV.

2.5. Interaction analysis

The interaction analysis was based on the Chou-Talalay method (Chou and Talalay 1984). The use of this approach for the study of the combined toxicity of mycotoxins has been described elsewhere (Alassane-Kpembi et al. 2013; Alassane-Kpembi et al. 2016). In brief, the gene activation dose-response relationships for the individual mycotoxins and their mixture were biometrically modeled 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. ED_{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 linear regression correlation coefficients of the median effects plots were greater or equal to 0.95 (Chou 2011).

Interactions were analyzed by plotting the isobolograms and calculating the combination-index (CI) values for the DON and NIV combination (Chou 2006):

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

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

For the genes exhibiting a synergistic up-regulation, the Dose Reduction Index (DRI) values indicated the folds of dose reduction for both DON and NIV. DRIs can be obtained from the reciprocal of each terms of the CI equation (Chou 2011):

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

and

$$(DRI)_1 = \frac{(D_x)_1}{(D)_1} \quad ; \quad (DRI)_2 = \frac{(D_x)_2}{(D)_2}$$

The dose-response relationship analysis for mycotoxins individual and combined effect for gene activation, the CI and DRI values and their 95% confidence intervals calculation, and f_a-CI plots for combined effects were performed with Compusyn software version 3.0.1 (ComboSyn Inc., Paramus, NJ, USA) (Chou and Martin 2005).

3. Results

3.1 DON and NIV induce an intestinal inflammatory response

The pro-inflammatory activity of DON and NIV on intestinal explants was assessed by measuring the gene expression for the chemokine IL-8 and the pro-inflammatory cytokines IL-1α, IL-1β, IL-17A and IL-22. After 5 hours of exposure, both mycotoxins induced an up-regulation of all tested genes (Fig 2). Explants exposed to the graded concentrations of DON and NIV (0.3-40 µM) showed a 5- to 10-fold increase expression of IL-1α and IL-8, while the increase for the pro-inflammatory cytokines IL-1β, IL-17A and IL-22 reached 20- to 40-fold (Fig. 2).

The pro-inflammatory genes demonstrated a bell-shaped regulation, as the upward trend of the gene activation for 10-20 µM levels of the mycotoxins was followed by a plateau or a decrease for higher concentrations.

For each mycotoxin and each pro-inflammatory gene, the highest exposure level that caused no detectable alteration of gene expression is presented in Table 2. For DON, the no effect doses ranged from 0.6 µM for IL-8, IL-17A and IL-22 to 1.25 µM for IL-1β and 5 µM for IL-1α. For NIV, the no effect dose was 2.5 µM for all tested cytokines/chemokine. We considered the lowest no effect doses of DON and NIV for all tested cytokines/chemokine as their No Observed Adverse Effect Levels (NOAELs) for the induction of intestinal inflammation. Thus the NOAELs reported for DON and NIV were 0.6µM and 2.5µM respectivelyA benchmark dose analysis was also performed, using both exponential and Hill dose-response models. Examples of the selected fitting models for the mRNA expression data are provided in supplemental material (Fig S1). The lower bounds of the 95% confidence interval of the mycotoxin concentrations required to cause an increase in mRNA expression one standard deviation above the control mean (BMDLs) are presented in Table 2. Among the tested cytokines, IL-22, IL-1β and IL-8 demonstrated the highest sensitivity to trichothecenes, and DON showed higher potency than NIV. Indeed, for DON, the BMDL ranged from 0.16 µM for IL-22 to 1.86 µM for IL-1α. For NIV they ranged from 0.73 µM for IL-22 to 4.11 µM for IL-17A.

3.2 Synergistic effect of DON and NIV on the induction of an intestinal inflammation.

DON and NIV often occur together, thus the next step was to characterize the type of interaction (additivity, synergy or antagonism) for their combined inflammatory effect. The dose-response curves generated for the individual and combined effect on IL-1α, IL-1β, IL-8, IL-17A and IL-22 gene expression were analyzed using the isobologram and combination-index method.

The DON-NIV combination demonstrated a dose-dependent up-regulation of the inflammatory response (Fig. 2). A bell-shaped activation pattern could be noted for all DON and NIV mixture dose-effect curves. The dose-effect curve parameters (*Amax*, D_m and *m*) for IL-1α, IL-1β, IL-8, IL-17A and IL-22 genes activation by the single mycotoxins and their mixture are presented in Table 3.

The isobologram for IL-1β activation by the DON and NIV combination at three different gene activation levels (25%, 50% and 75%) is presented in Fig. 3. 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 isobologram clearly revealed a synergy between DON and NIV at 25%, 50% and 75% activation levels for IL-1β. The synergistic effect of DON and NIV on intestinal IL-1β synthesis was also demonstrated through the calculation of combination-index. Indeed, whatever the fraction affected i.e. mRNA activation level, DON and NIV combinations give a CI below 1 (Fig 4). The CI values were also calculated for the other cytokines genes, IL-1α, IL-8, IL-17A and IL-22 over the whole range of activation levels. As shown in Fig 4, the DON-NIV combination showed synergistic effects for the activation of all the tested pro-inflammatory genes in the whole range of f_a values, with CI values clearly below 1. Moreover, the synergy increased with the mycotoxin concentrations. A step-by-step description of the dose-response data analysis for interaction assessment and the Compusyn® analysis report for IL-1β are provided in supplemental materials (Fig. S2).

In order to quantify the magnitude of the synergistic interaction, dose reduction indices (DRIs) were also calculated for three representative gene activation levels (AC_{25}, AC_{50} and AC_{75}). The DRI is the fold of dose that can be reduced for each

mycotoxin in the combination, for a given effect level, when compare with the dose of each mycotoxin alone for the same effect level. It denotes the factor by which the dose of each mycotoxin in the combination is reduced due to synergy. For the different tested inflammatory cytokines, the DRI varied from 2 to 11.The strongest synergistic effect was observed for IL-1β (Table 4). For this later cytokine, at 75%-activation, the DON-NIV mixture was 7-time more effective than DON alone, and 10-time more effective than NIV alone. The increased DRI observed for 25, 50 and 75% activation indicates that the magnitude of synergy increased with mycotoxin concentration.

4. Discussion

The intestine, that is the first barrier against food contaminants, shows high sensitivity to DON and related mycotoxins (Maresca 2013; Pinton and Oswald 2014. A survey in the United Kingdom estimated that 98% of the adults had been exposed to DON (Turner et al. 2008). Although DON often co-occurs with NIV in grains and grain products, the data describing their combined effects are lacking (EFSA 2013b). Our laboratory recently reported the ability for DON to disrupt the intestinal homeostasis of the inflammatory response (Cano et al. 2013). Indeed, exposure of a fully differentiated-three dimensional jejunal explant to DON induced an early intestinal inflammatory response resulting from the interplay of different cell types including the Th17 cells. Here we explored the dose-effect relationship for this inflammatory response upon exposure to realistic dietary concentrations of DON and NIV, and we analyzed their combined effects in terms of additivity, antagonism or synergy.

In a context of the 3Rs (replace-reduce-refine) the intestinal explant culture approach was chosen as it reduces the number of animals used while keeping enough strength for biological conclusion (Lucioli et al. 2013). Each pig allowed multiple doses assessment for DON, NIV and their mixture. The whole experiment (9 data-points x 3 mycotoxin/mixture conditions) was achieved with only 6 animals while the same statistical power would require more than 150 animals.

The tested concentrations ranged from 0.3 µM to 40 µM. A health-based guidance value (HBGV) has been established for DON from a NOAEL of 0.1 mg/Kg BW/day (JECFA 2011). Assuming a human adult of 70 Kg and a small intestinal volume of 1L, the NOAEL for DON would correspond to a mycotoxin concentration in the intestinal lumen of 21 µM (Maresca 2013).

The individual and combined pro-inflammatory activities of DON and NIV were analyzed by measuring the gene expression for the chemokine IL-8 and the pro-inflammatory cytokines IL-1α, IL-1β, IL-17A and IL-22. The ability of trichothecenes to induce immune- and inflammation-associated genes is well established and two mechanisms, binding to transcription factors and mRNA stabilization, have been proposed (Cano et al. 2013; Pestka 2010b). A clear bell-shaped activation of IL-1β and IL-8 by DON and NIV was observed. This biphasic effect was previously reported in human intestinal epithelial cells, with DON doses up to 25 µM causing a massive increase of IL-8 secretion whereas higher doses presented inhibitory effects (Maresca et al. 2008). This may be linked to the feed-back mechanisms downscaling pro-inflammatory gene effects that have been described: (i) the induction of suppressors of cytokine signaling (SOCS) (Amuzie et al. 2009), and (ii) the up-regulation of mitogen-activated protein kinase phosphatase 1 (MKP1) (Kinser et al. 2004).

To determine points of departure for the pro-inflammatory activity of DON and NIV, Benchmark dose analysis of genomic data was used in addition to the NOAEL approach. The primary advantage in applying genomic technology to risk assessment is the sensitivity of the molecular changes resulting from chemical exposure (Thomas et al. 2011). In the health-risk assessment, the NOAEL approach is a usual first step in the determination of guidance values. However, BMDL is gaining increasing consideration from regulatory agencies as it provides a better use of dose-response information, more appropriate reflection of experimental size and the lack of constraint to experimental doses (Filipsson et al. 2003). The NOAEL and BMD risk characterization approaches for non-cancer endpoints are based on the assumption of monotonic evolution of effect as a function of dose, which is being challenged by the unveiling of the non-monotonic U-shape or bell-shape dose-response relationships collectively referred to hormesis phenomenon (Calabrese and Baldwin 2003). The high end of the bell-shape regulation of the inflammatory genes observed in this experiment for individual as well as combined mycotoxin exposure could actually reflect a yet linked but quite distinct biological process (from the activation of pro-inflammatory genes *per se*) that is the onset of the downscaling mechanisms triggered by the suppressors of cytokine signalling (SOCS) and the mitogen-activated protein kinase phosphatase 1 (MKP1) (Amuzie et al. 2009; Kinser et al. 2004). From that perspective, NOAEL and BMD could apply to the intestinal pro-inflammatory effects of the trichothecene mycotoxins DON and NIV taking only into account the first part of the dose response curve.

The BMDLs we established for DON for the up-regulation of the pro-inflammatory cytokine IL-1β and IL-22 in the intestine were clearly below the micromolar range. The sensitivity of the inflammatory response compared to other effects exerted by DON has previously been suggested. Administrating DON to mice at doses that did not provoke any apparent adverse effects, modified their inflammatory status (Tardivel et al. 2015). The authors reported a systemic increase in plasma IL-1β concentration that correlated with an up-regulation of pro-inflammatory gene (IL-1β, TNF-α) mRNA expression in peripheral organs and brain structures in a time and concentration-dependent way. Since the gastrointestinal tract constitutes the first exposed organ to the food-borne toxins, it seemed relevant to monitor inflammation in this tissue. Intestinal concentrations of DON higher than 1µM in *in vitro* and *in vivo* experiments in animal and human have been associated to impaired nutrient absorption, decreased mucus production, as well as loss of barrier function and subsequent increase in bacterial translocation (Maresca 2013). Our results indicate that intestinal inflammation is a more sensitive endpoint, as a response was observed at 0.16 µM.

As DON and NIV are frequently reported to occur together, the design of this study also aimed to characterize the type of interaction (additivity, synergy or antagonism) for their combined inflammatory effect. The combination index method applied to transcriptomic data generated *ex-vivo* in the intestine, allowed us to analyze the combined pro-inflammatory effect of DON and NIV. We demonstrated that both mycotoxins act synergistically. To the best of our knowledge, this is the first application of the Median-Effect Principle (Chou and Talalay 1984) to genomic data. Unlike the biological inhibition processes that have been broadly investigated by the MEP principle, its application to the gene activation data required besides the median-effect dose (D_m)

and the shape of the dose-response curve (*m*) a third dose-response parameter, the maximal gene expression ratio (Amax) induced by the mycotoxins or their mixture.

The synergistic DON-NIV mixture was 2 to 14-time more potent than DON or NIV alone for inflammatory gene activation. The magnitude of interaction varied according to mycotoxin mixture concentration and the tested cytokine. A synergistic toxicity between DON and NIV has already been reported in a yeast bioassay (Madhyastha et al. 1994) and for DNA synthesis inhibition in mouse fibroblasts L929 (Tajima et al. 2002). We also previously reported a synergistic interaction for the *in vitro* cytotoxic effect exerted by low doses of DON and NIV toward human and porcine intestinal epithelial cells (Alassane-Kpembi et al. 2013; Alassane-Kpembi et al. 2015). Compared to the over-simplified biological models represented by the yeast bioassays and the cell culture systems, the synergistic toxicity evidenced for DON and NIV mixture in this study using the three-dimensional tissue explant model supports stronger extrapolation to whole organism. This organ-toxicity testing strategy was intended as a surrogate approach to traditional *in vivo* experiments, considering the minimal experimental group-size constraints for interaction analysis studies (Alassane-Kpembi et al. 2016). A drawback of using the explants model to investigate the pro-inflammatory effects of xenobiotics is the possible inflammatory/immune response induced by the explant surgery that could result in signal interfering or masking the specific effect of the tested stressors (Randall et al. 2011). Nonetheless, the differential expression of the inflammatory cytokine genes in mycotoxin-exposed explants compared to control ones in this experiment indicates that the impact of the surgery is minor or at least, not strong enough to mask the mycotoxin-induced inflammatory response.

The involvement of cellular active transport systems could be proposed to explain the differences in individual toxicity of DON and NIV and the pattern of toxicological interactions reported in this study. We hypothesize that the involvement of efflux proteins in the cellular trafficking of DON and NIV (Tep et al. 2007; Videmann et al. 2007) and possible differences in affinity for both mycotoxins to transporters could lead to intracellular accumulation of one or the other mycotoxin, especially NIV which presents a limited bioavailability by its own (Hedman et al. 1997; Poapolathep et al. 2003). This hypothesis still deserves to be further investigated. Nonetheless the implications of this synergistic interaction are of primary importance in terms of public health. In many European countries, several population groups are considered at risk, due to the narrow margin between their level of DON exposure and the toxicological threshold (EFSA 2013a). The ratio of safety threshold to the mean lower bond of the DON exposure level varies roughly from 2 in toddlers to 4 in adults and elderly. We showed that the toxicity enhancement resulting from the combined effects of DON and the frequently co-occuring NIV was from 2 to 14-times as potent as the toxicity of DON or NIV alone, which largely encompasses the safety margin for DON exposure in any of the European consumer population groups. Thus this study demonstrates that not taking into account the DON-NIV co-contamination of food could readily result in biases in risk analysis.

In conclusion, we analyzed the intestinal inflammatory response following individual or combined exposure to the co-occuring trichothecenes mycotoxins DON and NIV. Though the data obtained with this *ex-vivo* model still deserve to be confirmed *in vivo*, the results presented here are biologically relevant and highlight that very low doses of trichothecene significantly increase the expression of inflammatory genes in the intestine, and the combined pro-inflammatory activity of DON and NIV is synergistic. They also suggest the need for a reevaluation of the health risk associated to dietary exposure to trichothecene mycotoxins taking into account more sensitive endpoints and combined exposure patterns.

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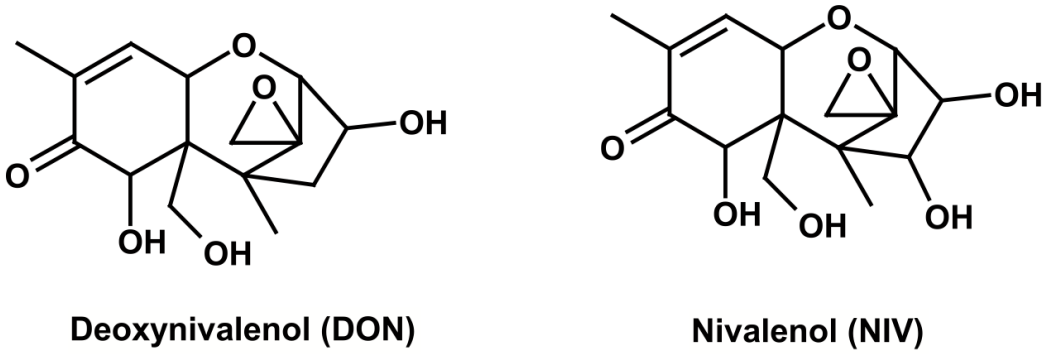
Figure 1: Chemical structures of DON and NIV



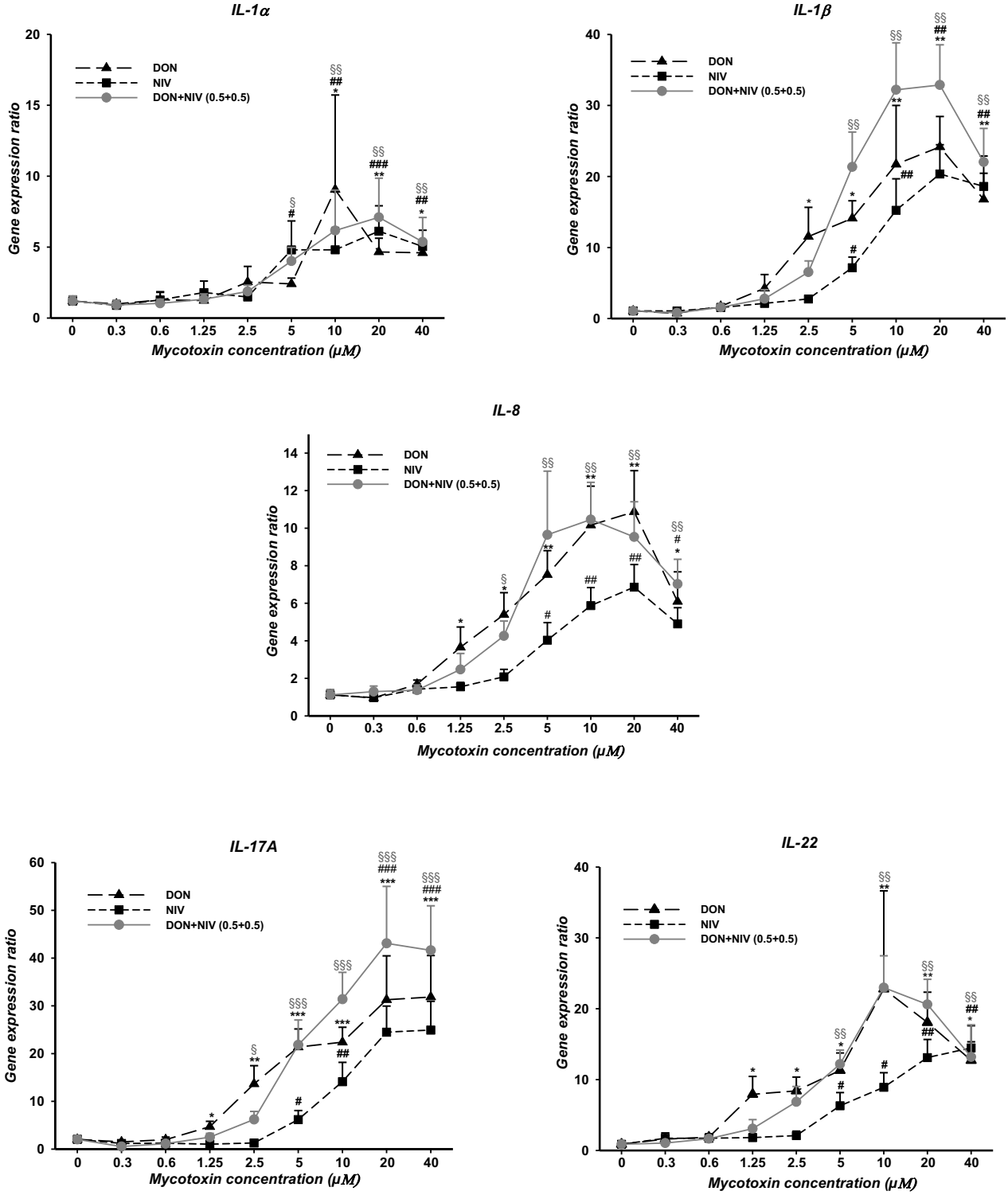


Figure 2: DON, NIV and their mixture (0.5:0.5) dose-responses for the regulation of IL-1α, IL-1β, IL-8, IL-17A and IL-22 expressions in the pig intestine. Jejunal explants from 6 piglets were exposed for 5 hours to graded levels of the individual trichothecene mycotoxins or their mixture. The amounts of the target mRNAs were measured by quantitative RT-PCR and analyzed using REST© 2009 software (Qiagen, Valencia, CA). *β2-Microglobulin* and *RPL-32* were used as the internal references. Data are means ± SEM (n=6 animals). Symbols *, **, *** or $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ or §, §§, §§§ indicate statistical significant differences in gene expression ratios for treated conditions compared to the control ($p<0.05$, $p<0.01$, $p<0.001$ respectively)

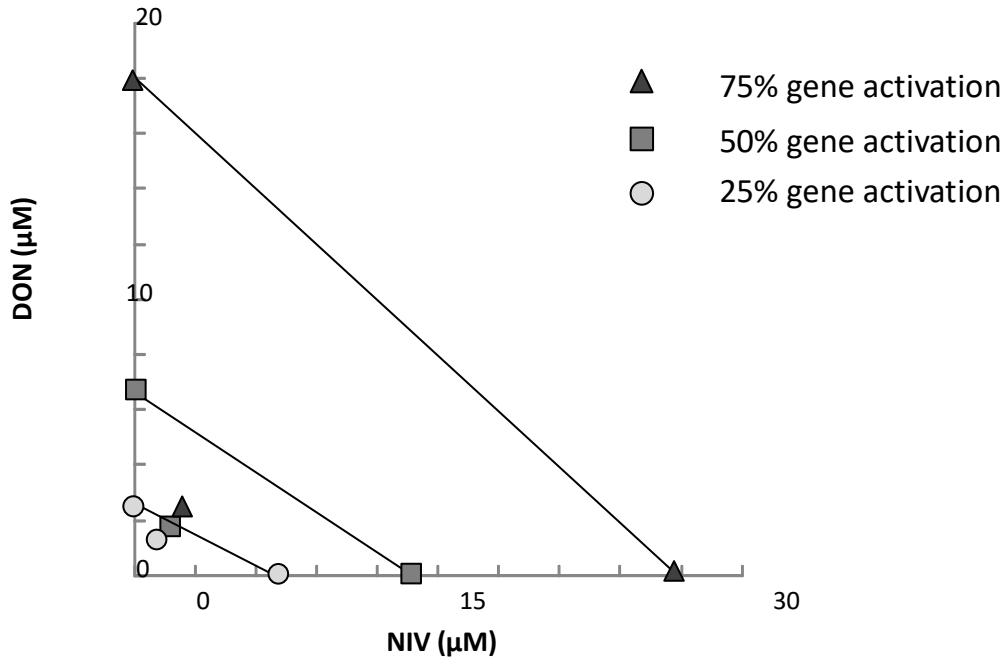


Figure 3: Isobologram illustrating the combined activation of IL-1β by DON and NIV at concentrations eliciting 25% (●), 50% (■) or 75% (▲) gene activation. The points are mean concentrations of dose-response curves generated from explants obtained from 6 piglets for each mycotoxin or their combination (CompuSyn software analysis (Chou and Martin 2005)). See CompuSyn report given in Supplementary Printout (Fig. S1).

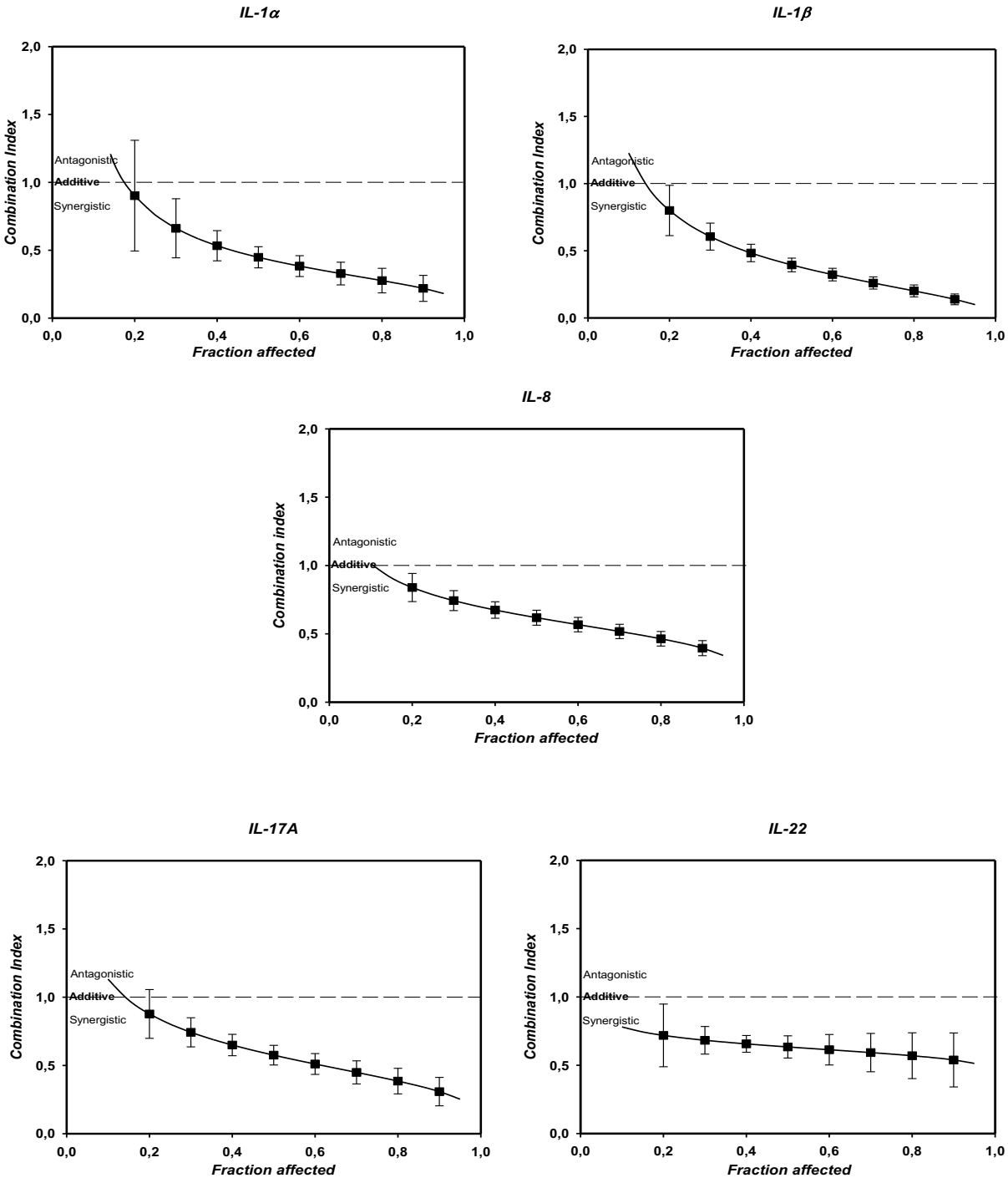


Figure 4: Combination index – fraction affected curves for IL-1α, IL-1β, IL-8, IL-17A and IL-22 expressions in the pig intestine following exposure to the DON-NIV combination. The fraction affected is the fractional up-regulation of gene expression induced by a given dose of single mycotoxin or mixture. CI values were calculated from gene expression data obtained from 6 piglets tissue cultures exposed for 5 hours to graded levels of the individual trichothecene mycotoxins or their mixture using CompuSyn software (Chou and Martin 2005). The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (Chou 2006)

Table 1: list of genes, primer sequences (F: Forward; R: Reverse) and accession numbers and references

Gene Symbol	Gene name	Primer sequence	Accession number and reference
IL-1α	Interleukin 1 - alpha	F: TCAGCCGCCCATCCAA	NC_010445.3 Cano et al. (2013)
		R: AGCCCCGGTGCCATGT	
IL-1ß	Interleukin 1 - beta	F: GAGCTGAAGGCTCTCCACCTC	NM_001005149 Cano et al. (2013)
		R: ATCGCTGTCATCTCCTTGCAC	
IL-8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC	NM_213867 Bracarense et al. (2012)
		R: AAGGTGTGGAATGCGTATTTATGC	
IL-17A	Interleukin 17 - alpha	F: CCAGACGGCCCTCAGATTAC	NM_001005729.1 Pierron et al. (2016b)
		R: GGTCCTCGTTGCGTTGGA	
IL-22	Interleukin 22	F: AAGCAGGTCCTGAACTTCAC	AY937228 Cano et al. (2013)
		R: CACCCTTAATACGGCATTGG	
RPL32	Ribosomal Protein L32	F: AGTTCATCCGGCACCAGTCA	NM_001001636 Pinton et al. (2010)
		R: GAACCTTCTCCGCACCCTGT	
β2-Microglob	β2-Microglobulin	F: TTCTACCTTCTGGTCCACACTGA	L13854 Grenier et al. (2011)
		R: TCATCCAACCCAGATGCA	

Table 2: No effect doses and Benchmark doses for the intestinal up-regulation of the pro-inflammatory cytokines gene expression by DON and NIV

Cytokine	**Mycotoxin**	***No effect dose (µM)***	***BMDL (µM)***
IL-1α	DON	*5*	*1.86*
	NIV	*2.5*	*1.74*
IL-1β	DON	*1.25*	*0.51*
	NIV	*2.5*	*0.87*
IL-8	DON	*0.6*	*0.54*
	NIV	*2.5*	*1.84*
IL-17A	DON	*0.6*	*0.51*
	NIV	*2.5*	*4.11*
IL-22	DON	*0.6*	*0.16*
	NIV	*2.5*	*0.73*

Pig jejunal explants were exposed for 5 hours to graded levels of DON or NIV. The PROAST software package Version 38.9 that has been developed by the Dutch National Institute for Public Health and the Environment was utilized for the calculation of the BMDL values. Dose-response data of the pro-inflammatory cytokines mRNA expression levels in the explants were fit using exponential and Hill continuous models. BMDLs refer to lower 95% confidence limit of the corresponding BMDs that are the mycotoxin concentrations required to cause an increase in mRNA expression one control standard deviation above the control mean.

Table 3: Activatory dose-effect relationship parameters for DON, NIV and their combinations on the gene expression of pro-inflammatory cytokines

Cytokine	DON			NIV			DON+NIV (1:1)		
	*A*max*	D_m[§]	*m*[†]	*A*max	D_m	*m*	*A*max	D_m	*m*
IL-1α	4.65	15.66	1.25	6.12	7.89	1.17	7.10	4.21	2.13
IL-1β	24.15	6.73	1.11	20.35	13.61	1.63	32.88	3.55	3.27
IL-8	10.87	2.73	1.79	6.86	12.32	1.22	10.46	2.76	2.54
IL-17	31.85	7.58	1.24	24.93	17.46	1.73	43.09	6.08	2.27
IL-22	22.79	4.67	1.52	14.47	19.75	1.11	22.98	4.79	1.61

* Maximal gene expression ratio induced by mycotoxins (*A*max)

[§]D_m is the median-activation concentration or the half-maximal activation concentration

[†]*m* is the slope of median-effect plot signifying the dynamic order or the shape of dose-activation curve, where *m*=1, >1, and <1 indicates hyperbolic, sigmoidal, and flat sigmoidal shape, respectively.

Table 4: Combination and Dose reduction index values for DON and NIV combination (1:1) on the activation of pro-inflammatory cytokines gene expression

Cytokine	CI* at				DRI[§] at		
	AC_{25}	AC_{50}	AC_{75}		AC_{25}	AC_{50}	AC_{75}
IL-1α	0.62	0.45	0.27	DON	5.19	7.44	10.66
				NIV	2.46	3.75	5.72
IL-1β*	0.69	0.39	0.23	DON	1.98	3.79	7.27
				NIV	5.46	7.66	10.74
IL-8	0.79	0.62	0.49	DON	1.64	1.98	2.37
				NIV	5.6	8.92	14.2
IL-17A	0.8	0.58	0.42	DON	1.67	2.49	3.73
				NIV	4.94	5.74	6.67
IL-22	0.7	0.63	0.58	DON	1.87	1.95	2.03
				NIV	6.05	8.24	11.22

*Combination Index (CI) indicates synergism (CI<1), additive effect (CI=1) and antagonism (CI>1) of the activation of pro-inflammatory cytokines gene expression at 25%, 50% and 75% activation concentrations. CI values were calculated using CompuSyn software (Chou and Martin 2005). For IL-1β see CompuSyn report given in Supplementary Printout.

[§]Dose-reduction index (DRI) indicates fold of dose reduction allowed for a given activation level (e.g. 25%, 50% and 75% activation) of gene expression in combination of DON (1st entries) and NIV (2nd entries) when compared to each mycotoxin alone. The greater DRI (>1) indicate more dose reduction.