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The intestinal toxicity of mycotoxins: analysis of the interactions between type B trichothecenes

Imourana Alassane-Kpembé

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Université
de Toulouse

THÈSE

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Once upon a time, there was a Master who held two bottles of antitumor ingredients. The red bottle contained drug A, and the blue bottle contained drug B. He then gave the two bottles to his disciples, John and Paul. The Master asked them to conduct drug combination studies in different proportions and to determine whether they were synergistic or antagonistic by using any of their best choice of assay method and by using the best choice of theory for their dose-effect data analysis...

Chou TC Pharmacol Rev. 2006; 58(3):621-81

The Master waited several years until I met him somewhere between York Avenue and 1st Avenue, while looking for my way in that very misleading field of drug combination studies, then he retired. Thanks so much !

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RESUME

L'intestin est la première barrière de l'organisme contre les contaminants alimentaires, dont les mycotoxines. Le déoxynivalénol (DON) est un contaminant majeur des céréales, souvent retrouvé en association avec d'autres trichothécènes B (TCTs B), le 3- et 15-acétyldéoxynivalénol (3-ADON et 15-ADON), le nivalénol (NIV) et la fusarénone X (FX). Au niveau cellulaire, le DON interagit avec l'ARN ribosomique, bloquant ainsi la synthèse protéique et activant la cascade de la voie de signalisation de MAPKinases impliquée dans des mécanismes de la réponse inflammatoire. Au niveau intestinal, cette mycotoxine pourrait donc perturber le renouvellement continu de l'épithélium, et l'homéostasie de la réponse inflammatoire. On suggère ainsi qu'elle pourrait jouer un rôle dans la pathogénie des maladies inflammatoires chroniques de l'intestin. Si les effets du DON sont relativement connus, ceux du NIV et de leurs dérivés acétylés sont moins bien documentés. De même, peu de données existent quant à la toxicité combinée de ces mycotoxines dont la co-occurrence est avérée.

Sur des modèles *in vitro* de cellules épithéliales intestinales humaines et porcines et sur un modèle *ex vivo* d'explants de jéjunum de porc, nous avons comparé les toxicités individuelles de cinq TCTs B (DON, 3- et 15-ADON, NIV et FX) et analysé leur toxicité combinée en termes de synergie, additivité ou antagonisme vis-à-vis de l'intestin.

Les résultats montrent qu'à des concentrations de l'ordre du micromolaire, les TCTs B inhibent la croissance des cellules épithéliales intestinales par ordre croissant de toxicité 3-ADON, DON, 15-ADON, NIV et FX. Aux faibles doses correspondant à des niveaux d'exposition rencontrés chez le consommateur français ou européen, des synergies d'un facteur 3 à 10 ont été observées. Ces travaux ont également permis de caractériser l'activité pro-inflammatoire au niveau intestinal des TCTs B, et l'analyse benchmark de données de transcriptomique a montré que l'exposition de l'intestin à des doses aussi faibles que 0.04 μ M de FX, 0.1 μ M de DON ou 0.1 μ M de NIV s'accompagne d'une activation significative des mécanismes de l'inflammation. Ces

doses sont de l'ordre des concentrations attendues dans le chyle sur la base des valeurs toxicologiques de référence actuelles. En conclusion, ces données montrent que le renouvellement de l'épithélium intestinal et l'activité pro-inflammatoire au niveau intestinal pourraient être des marqueurs très sensibles dans le cadre de l'évaluation de la toxicité individuelle et des interactions entre TCTs B.

ABSTRACT

As for other food-born contaminants, the gastro-intestinal tract represents the first barrier against deoxynivalenol (DON). This mycotoxin frequently co-occurs with other type B trichothecenes (TCTs B) namely 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon-X (FX). At the cellular level, DON binding to ribosomal RNA results in the inhibition of protein synthesis and triggers the mitogen-activated protein kinases (MAPKs) pathway that have been linked to immune response mechanisms. Thus, intestinal epithelial cell renewing is considered a putative target in DON toxicity. Moreover, based on the ability of DON to disturb the state of homeostasis of the inflammatory response in the intestine mimicking what is found in inflammatory bowel diseases (IBD), it is proposed that this mycotoxin may play a role in such diseases. However, very few is known about the intestinal toxicity of the other co-occurring TCT B, and their combined effects eventually.

By means of *in vitro* human and porcine intestinal epithelial cells models and an *ex vivo* porcine jejunal explants model, we assessed the individual toxicity of five TCT B (DON, 3- and 15-ADON, NIV and FX) toward the intestine and we analyzed their combined toxicity in terms of additivity, synergy or antagonism.

The tested TCT B significantly impaired the intestinal epithelial cell growth in the micromolar range, in increasing order of potency 3-ADON, DON, 15-ADON, NIV and FX. The toxicity of low doses of TCT B was synergistic. For mycotoxin concentrations corresponding to exposure levels reported for French and European consumers, the amplitude of this synergy ranged between 3 and 10. Benchmark dose analyses of the transcriptional data also showed that the exposure of the intestine to mycotoxin concentrations as low as 0.04 μ M for FX, 0.1 μ M for DON and 0.1 μ M for NIV could be associated to a significant activation of the inflammatory response mechanisms. Taken together, these results suggest that epithelial cell renewing and pro-inflammatory effects at the intestinal level may be consider very sensitive

biomarkers for the assessment of the individual toxicity and interactions between the co-occurring TCTs B.

Liste des publications et communications:

Articles

Alassane-Kpembé, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F.A., Oswald, I.P., Puel, O., 2013. New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. *Toxicol Appl Pharmacol* **272**, 191-198.

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Alassane-Kpembé I., Puel O., and Oswald I.P. 2013. Toxicological interactions between deoxynivalenol, nivalenol and their acetyl derivatives in intestinal epithelial cells. *Soumis pour publication à Toxicology*.

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cellules épithéliales intestinales. Congrès de la Société de Pharmacotoxicologie Cellulaire, Toulouse (France), 19 – 20 Mai 2011. (*Prix du meilleur poster*)

Alassane-Kpembé I., Gauthier T., Lyazrhi F., Puel O., Abiola F. A., Kolf-Clauw M. et Oswald I.P. Interactions des trichothécènes B : Evaluation de la cytotoxicité combinée du DON et de ses dérivés acétylés. Journées d'animation scientifique du département de Santé Animale INRA, Fréjus (France), 22 – 25 Mai 2011.

Liste des abréviations

μM : Micromolaire	IL : Interleukine
15-ADON : 15-acetyldéoxynivalénol	IPEC-1 : Intestinal Porcine Epithelial Cells-1
3-ADON : 3-acetyldéoxynivalénol	JNK : Jun N-terminal kinase
AFB ₁ : Aflatoxine B ₁	kDa : Kilo Dalton
BMD : Benchmark dose	MAPK : Mitogen-activated protein kinase
BMDL : Benchmark dose Lower bound confidence limit	MRP-2 : Multidrug resistance-associated protein 2
CI : Combination index	MTT : 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide
COX : Cyclo-oxygénase	NF- κ B : Nuclear factor kappa-B
CPA : Cyclopiazonic acid	NIV : Nivalenol
DJT : Dose journalière tolérable	NOAEL : No-observed adverse effect level
D_m : Median-effect dose	NOD : Nucleotide-binding oligomerization domain
DON : Déoxynivalenol	NR : Neutral red
DRI : Dose reduction index	OMS : Organisation mondiale de la santé
ERK : Extracellular signal-regulated protein kinase	OTA : Ochratoxine A
f_a : fraction affected	PODs : Points of Departure
FAO : Food and agriculture organization	RT-PCR : Reverse transcriptase polymerase chain reaction
FB ₁ : Fumonisine B ₁	SCF : Scientific committee on food
FX : Fusarénone-X	SOCS : Suppressor of cytokines signaling
IBD : Inflammatory bowel disease	TCT : Trichothécène
IC ₅₀ : Inhibitory concentration 50%	Th1 et Th2 : Lymphocytes T-auxiliaire (helper) 1 et 2
IFN : Interféron	TLR : Toll-like receptor
Ig : Immunoglobuline	TNF- α : Tumor necrosis factor α
	ZEA : Zéaralénone

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I. INTRODUCTION

1. CONTEXTE DE L'ETUDE

Les moisissures, en particulier celles du genre *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* et *Alternaria*, qui colonisent les denrées végétales (céréales, fruits, noix, amandes, grains, fourrages) au champ ou en cours de stockage produisent une multitude de métabolites secondaires. La sécrétion de ces composés, de structures chimiques très variées, vise entre autres à faciliter aux moisissures la colonisation du substrat, en leur procurant par exemple un avantage par rapport autres micro-organismes avec lesquels ils seraient en compétition. Au nombre de ces métabolites secondaires, certains qui présentent une toxicité plus ou moins marquée pour les humains et les animaux sont appelés mycotoxines. On estime que 25% des récoltes mondiales véhiculent ces contaminants naturels dont la plupart ne sont pas dégradés par la chaleur et restent présents même après la disparition des champignons (CAST, 2003). Les principales classes de mycotoxines aujourd'hui recensées sont les aflatoxines, les trichothécènes (TCTs), les fumonisines, les ochratoxines, les alcaloïdes de l'ergot de seigle et la zéaralénone.

En France, comme dans les grands bassins de production de céréales dans le monde, les conditions climatiques sont propices au développement des champignons du genre *Fusarium*, et en particulier *F. graminearum* et *F. culmorum*. Ces céréales sont ainsi susceptibles d'être contaminées par du déoxynivalenol (DON), une mycotoxine du groupe des TCT. D'un enjeu de santé publique au départ restreint à la niche écologique des espèces de champignon productrices, l'accroissement du volume des échanges commerciaux internationaux et la place des céréales dans ces échanges, ont fait de la contamination des aliments par le DON une préoccupation mondiale. Depuis 2010, le DON se classe ainsi en tête de peloton des mycotoxines les plus fréquemment rencontrées dans le monde, avec plus de 60% des échantillons d'aliment testés positifs (Schatzmayr et Streit, 2013).

Outre le DON, *F. graminearum* et *F. culmorum* produisent d'autres mycotoxines telles que la zéaralénone, la fusarine C, la chrysogine ainsi que de nombreux autres TCT B (15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), nivalenol (NIV) et fusarénone X (FX), mais aussi des toxines dont la structure reste à élucider. On estime que seulement 20% des métabolites de *Fusarium* sont identifiés structurellement. En conséquence, l'homme et l'animal ne sont généralement pas exposés à une mycotoxine, mais à un cocktail de toxines en même temps. De nombreuses enquêtes menées à l'échelle mondiale montrent ainsi que la co-contamination par plusieurs mycotoxines est la règle plutôt que l'exception (Rodrigues et Naehrer, 2012; Streit *et al.*, 2012; Streit *et al.*, 2013). Il est légitime de s'interroger sur le risque cumulatif associé à cette exposition simultanée à plusieurs toxiques, et ses implications sur l'innocuité d'aliments contaminés à des niveaux jugés acceptables pour chaque toxique pris individuellement. La plupart des études de toxicité ayant analysé les effets des mycotoxines individuellement, peu d'éléments existent à ce jour pour une évaluation du risque qui prenne en compte de possibles effets toxiques additifs, synergiques, voire antagonistes. Il est donc important d'étudier les effets des co-contaminations mycotoxiques pour leur prise en compte par la réglementation.

Le porc est particulièrement exposé aux mycotoxines, du fait de son alimentation qui est essentiellement composée de céréales. Contrairement aux polygastriques doués de bonnes capacités de détoxification d'un certain nombre de mycotoxines, le porc est une espèce particulièrement sensible. Il est également très similaire à l'humain du point de vue transcriptomique, protéomique et immunitaire. A ce titre, il représente un bon modèle d'étude de l'impact des mycotoxines contenues dans les aliments sur la santé (Nejdfors *et al.*, 2000).

Dans le tractus gastro-intestinal la biodisponibilité des mycotoxines, comme celles des autres contaminants alimentaires, est maximale, et l'intestin représente la première cible, mais également la première ligne de défense de l'organisme contre ces toxiques. Chez les mammifères adultes l'intestin est l'organe qui présente le pouvoir de

régénération le plus élevé, et on estime que l'épithélium intestinal est entièrement renouvelé entre 72h et 96h (Heath, 1996). Il est, de fait, très sensible à la toxicité du DON et des autres mycotoxines du groupes des TCT qui sont des inhibiteurs connus de la synthèse protéique (Ueno, 1985).

Notre projet de recherche ayant abouti à la rédaction de cette thèse dans le cadre de la préparation du Doctorat de l'Université de Toulouse, avait pour objectif de caractériser la toxicité individuelle et combinée de cinq TCT B, le DON, le NIV et leurs dérivés acétylés (3-ADON, 15-ADON et FX) vis à vis de l'intestin, en utilisant deux modèles de complexité croissante:

- (i) Le renouvellement de l'épithélium intestinal
- (ii) La réponse inflammatoire intestinale induite par ces mycotoxines.

2. ETUDE BIBLIOGRAPHIQUE

La synthèse bibliographique se présente sous la forme d'un chapitre de livre consacré à l'effet des contaminants alimentaires notamment les mycotoxines sur la santé intestinale, et d'un article de revue qui traite des différentes approches méthodologiques et des conclusions des études *in vitro* des interactions toxicodynamiques entre mycotoxines.

L'intestin est particulièrement exposé aux contaminants aussi bien naturels que d'origine anthropogénique qui peuvent être véhiculés par les aliments. Chez les animaux à cycle court et plus particulièrement les monogastriques, la santé intestinale est un déterminant majeur de l'efficacité alimentaire et donc des performances zootechniques enregistrés dans les élevages. De même, la physiologie de ces animaux est à bien des égards comparable à celle de l'homme. Partant des mycotoxines comme exemple de contaminant naturel et des dioxines comme exemple de contaminant d'origine anthropogénique, nous nous sommes intéressés dans la première partie de cette synthèse bibliographique aux effets des contaminants alimentaires sur les principales fonctions physiologiques de l'intestin rapportés dans des études de toxicologie ayant des monogastriques comme modèle d'étude.

L'intestin est très vraisemblablement soumis aux effets simultanés de plusieurs contaminants. La deuxième partie de synthèse aborde donc la complexité des approches méthodologiques dans les études d'interaction de type toxicodynamique des xénobiotiques en général, et de façon plus spécifique, les conclusions biologiques d'études consacrées à l'évaluation *in vitro* de la toxicité combinée des mycotoxines.

2.1. Effets des contaminants alimentaires sur la santé intestinale des animaux monogastriques

De par l'importance de sa surface d'échange avec le milieu extérieur, la muqueuse intestinale est particulièrement exposée à diverses agressions de nature chimique ou microbiologique. Les trois principales fonctions de cette muqueuse peuvent être affectées par les contaminants alimentaires. Elle sert d'abord de barrière physique entre le l'organisme et le contenu de la lumière intestinale. Elle est responsable de la digestion puis de l'absorption des nutriments. Elle sert enfin de métronome de la réponse immunitaire locale en participant à la mise en place de l'homéostasie nécessaire à l'orientation vers la tolérance aux antigènes associés à la flore commensale et aux aliments, et aux mécanismes actifs de défense mis en jeu en présence d'antigènes associés à la flore pathogène. Nous présentons dans cette revue les effets décrits pour deux types de contaminants alimentaires (les mycotoxines et les dioxines) sur ces fonctions. Cette étude fait la synthèse des modes d'action de ces deux classes de contaminants au niveau de l'intestin, et montre clairement que des niveaux d'exposition usuellement rencontrés sont susceptibles d'impacter négativement l'intégrité et la fonctionnalité de cet organe.

Cette revue rédigée sous la direction du Dr Isabelle OSWALD, a été acceptée comme chapitre de l'ouvrage intitulé "Intestinal health, key to optimise production" (Ed. Theo Niewold) *Wageningen Academic Publishers* à paraître en Janvier 2014.

Effect of feed contaminants on intestinal health of monogastric farm animals

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Abstract

As the most extensively exposed surface in the body, the intestinal mucosa has to face important chemical and biological challenges. The intestinal mucosa has three main physiological functions. It establishes a physical barrier between the internal milieu and the luminal content. The intestinal mucosa is also responsible for luminal nutrients digestion and their subsequent absorption. The mucosal epithelium is at the interface of immune system and luminal contents, including dietary antigens and microbial products. This implies a local defence mechanisms regulation that involves integrating all the signals that come from the external and internal world to preserve immune homeostasis steady-state conditions. Either of these intestinal physiological functions may be targeted by feed contaminants. These contaminants may be naturally occurring compounds or substances from anthropogenic sources. In the present chapter, we present mycotoxins and dioxins, which are representative examples of both classes of contaminants. Data gathered show clearly that dietary exposure to realistic doses of these contaminants impairs intestine functionality and its integrity as well. The mechanisms of action of mycotoxins and dioxins targeting the gastrointestinal tract are clarified and evidences for their deleterious effects for monogastrics intestinal health is provided.

Keywords: mycotoxins, dioxins, barrier function, absorption-digestion, immunity

I. Introduction

The gastrointestinal tract has the most extensively exposed surface in the body and is constantly exposed to potentially harmful food-borne substances from diverse sources (Yegani and Korver, 2008). Gut damages caused by these contaminants may lead to poor intestinal health. Three main functions that may be targeted by feed contaminants are devolved to the intestinal mucosa (Rescigno, 2011; Turner, 2009). First, it establishes a physical barrier between the internal milieu and the sometime hostile luminal content. The intestinal mucosa is also responsible for nutrients digestion and their subsequent absorption, which require a selectively permeable barrier. For these two functions, the mucosal epithelium is *de facto* at the interface of immune system and luminal contents, including dietary antigens and microbial products. That raises the third function for intestinal mucosa, which is in charge of integrating all the signals that come from the external and internal world to preserve intestinal immune homeostasis steady-state conditions.

Naturally occurring contaminants and pollutants from anthropogenic sources are the two classes of contaminants that may be present in feed. Natural contaminants from both plants and fungal specific primary and secondary metabolisms may be found in feed. These substances may have anti-nutritional or specific toxic effects for farm animals. Toxins from fungal and plant origin include and are not limited to mycotoxins, lectins, cyanogens, gossypol, glucosinolates and phyto-oestrogens (for a review see D'Mello 2004). Environmental naturally occurring contaminants like heavy metals and metalloids are widespread and can be found from traces to macro levels. Their significant contamination through soil of seeds and plants fed to animals may also result in chronic toxicity. Contaminants from anthropogenic source are in general substances manufactured for industrial use that are not naturally occurring, but may enter accidentally or deliberately the environment and result in environmental, agricultural, industrial or other contaminations. In other cases industrial activity may increase the mobility of naturally-occurring chemicals, or increase their amount

available to circulate in the environment, allowing them to contaminate feed at higher levels than would otherwise occur. Examples of these anthropogenic contaminants include persistent organic pollutants (especially dioxins), pesticides and radionuclides.

In this chapter, we present mycotoxins and dioxins which are illustrative examples of both feed contaminants classes, and their reported effects as well as mechanisms of action targeting the gastrointestinal tract and potentially deleterious for the intestinal health of monogastric farm animals.

Mycotoxins in Feed

Mycotoxins are secondary fungal metabolites produced under specific environmental conditions by a variety of mould spoiling agricultural commodities. As secondary metabolites, they are not essential for life, but may provide the fungus with an ecological advantage in certain environments. Factors contributing to the presence of mycotoxins include ecological and storage conditions that are most times beyond human control. Toxigenic molds are known to produce one or more of these mycotoxins and a substrate can be spoiled by more than one mold. Some 300 compounds have been recognized as mycotoxins of which a dozen is considered as threat to human and animal health. It has been estimated that at least 25% of the yearly worldwide grain production is contaminated (CAST, 2003). The mycotoxins of biological and economical significance in animal agriculture, *i.e.*, aflatoxins, fumonisins, trichothecenes, zearalenone, and ochratoxins have been extensively reviewed and will be only shortly described in this chapter (Bennett and Klich, 2003; Bryden, 2012).

Aflatoxins

Aflatoxins were isolated and identified in England in the earlier 1960's as the cause of a mysterious outbreak of hepatic necrosis affecting thousands of poultry. Similar incidents were then reported in pigs. Investigations revealed that toxicity was

associated to the presence of *Aspergillus flavus* in the feed and further that extracts of the culture of the fungus were capable of inducing the syndrome. Later many other species in the sections *Flavi*, *Nidulantes* and *Ochraceorosei* were also identified as aflatoxin-producers (for detailed review see Varga *et al.*, 2011). Structurally aflatoxins are difurocoumarin derivatives that fluoresce under ultraviolet light. The most important aflatoxin in terms of toxic potency and occurrence is aflatoxin B₁ which has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC). AFB₁ is hepatotoxic and hepatocarcinogenic, but many other effects can be associated to its toxicity: immunosuppression, reduced growth rate and reproductivity, lowered milk and egg production (Rawal *et al.*, 2010).

*Fumonisin*s

Fumonisin are products of polyketide and amino acid metabolism and have a linear structure with amine and tricarballic ester functions. They are produced by *Fusarium verticillioides* and many other *Fusarium* species. Some twelve fumonisins have been isolated, but fumonisin B₁ (FB₁) is the most abundantly produced and the most toxic. The toxicity of FB₁, including its effect on the intestine, is mainly exerted through the ability of this toxin to disrupt sphingolipid metabolism (Bouhet and Oswald, 2007). The IARC has classified FB₁ as possibly carcinogenic to humans (group 2B). This toxin may also be implicated in the aetiology of human oesophageal cancer and neural tube defects. FB₁ also causes leukoencephalomalacia in equines, pulmonary oedema and hydrothorax in swine, nephrotoxicity and hepatotoxicity in many species.

Trichothecenes

Trichothecenes are closely related sesquiterpenoid mycotoxins with a 12,13-epoxy ring and a variable number of hydroxyl, acetoxy or other substituents. They are classified as macrocyclic or non-macrocyclic depending on the presence of a macrocyclic ester or an ester-ether bridge between C-4 and C-15. Food and feed-borne

trichothecenes are mainly non-macrocyclic compounds produced by fungi of the *Fusarium* genus. They can be subclassified in type A which have a hydrogen or ester type side chain at the C-8 position and type B which have a ketone group instead. Trichothecenes are well-characterized inhibitors of the protein and nucleic acids synthesis. In acute toxicity tests, type A members such as T-2 toxin have been found to be more toxic than type B components such as deoxynivalenol (DON) and nivalenol (NIV). However in practical situations of chronic intake, the effects and syndromes arising from DON are likely to be more important. In animal studies, chronic exposure to trichothecenes caused impaired weight gain, anorexia, hematotoxicity and immune dysregulation.

Zearalenone

Zearalenone (ZEA) frequently co-occurs with certain type B trichothecenes as they are simultaneously produced by the same fungi. ZEA is a non-steroidal oestrogen and its alcohol metabolites (α -zearalenol and β -zearalenol) have increased oestrogenic activity corresponding to their binding affinities for hepatic, uterine, mammary and hypothalamic oestrogen receptors. Dietary exposure to low doses of ZEA leads to hyperoestrogenic syndroms and subsequent reproductive dysfunctions in pigs.

Ochratoxins

Ochratoxins are isolated from fungi belonging to *Aspergillus* and *Penicillium* geni. They are chemically described as 3,4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L- β -phenylalanine. The most commonly occurring and most toxic member is ochratoxin A (OTA) which toxicological profile includes nephrotoxicity, hepatotoxicity, teratogenicity and immunotoxicity. In addition, OTA has been demonstrated to be carcinogenic among laboratory animals, which justifies that IARC has rated OTA as a possible human carcinogen (group 2B). Among farm animals, pigs are particularly sensitive to the toxin for its tissue

accumulation, due to a rather long serum half-life and the entero-hepatic recirculation. The feed occurrence of ochratoxins is not only important from the animal health and performance perspective, but also from the potential human indirect exposure through the animal derived foods consumed.

Dioxins in feed

Dioxins and dioxin-like chemicals are structurally related and environmentally persistent compounds that share a common mechanism of action, and as a consequence a common spectrum of biological responses. The term “dioxin” commonly refers to polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) that are trace level unintentional by-products of most forms of combustion and several industrial chemical processes including chlorine bleaching of wood pulp and the manufacture of organochlorine pesticides, phenoxyacid herbicides and fungicides. PCDDs and PCDFs may also be present as contaminants in polychlorinated biphenyls (PCBs), which are considered dioxin-like compounds. While PCDDs and PCDFs are produced unintentionally as unwanted by-products, PCBs were manufactured for use in transformers, insulators, and many others technological applications. PCB production was banned by the Stockholm Convention on Persistent Organic Pollutants in 2001. Exposure levels are expressed in toxic equivalents (TEQ) of the most toxic congener, 2,3,7,8-tetrachloro-dibenzo-p-dioxin (2,3,7,8-TCDD). The main health issues concerning the dioxins have been reviewed elsewhere (Schechter *et al.* 2006). These compounds have a high-affinity binding to the aryl hydrocarbon receptor (AhR) which is an intracellular ligand-activated transcription factor involved in regulation of the expression of a large number of genes and which also interacts with regulatory proteins such as specific cellular kinases, and some of cell cycle control as well as apoptosis proteins. Even tiny doses of dioxins can cause death in certain laboratory animals and wildlife species, and 2,3,7,8-TCDD is considered the most toxic man-made chemical. Its LD₅₀ for guinea pigs is evaluated to 1µg/kg body weight. The observed effects in humans and experimental animals

include toxicity to the immune system, reproductive and developmental abnormalities, and endocrine disruption with diabetes and thyroid disorders (Bursian *et al.*, 2013a; b; Pavuk *et al.*, 2003; Weisglas-Kuperus *et al.*, 2000). The IARC has classified 2,3,7,8-TCDD as Group 1, carcinogenic to humans. Polygastrics grazing pastures that are close to industrial area are typically considered farm animals exposed to dioxin risk (Kamphues and Schulz, 2006), but the Belgian PCB/dioxin incident revealed that poultry and pigs may also be at risk (Bernard *et al.*, 2002; Covaci *et al.*, 2008). For example, an incident occurred at the end of January 1999 when a mixture of PCBs contaminated with dioxins was accidentally added to a stock of recycled fat used in the production of animal feeds. Signs of poultry poisoning were noticed by February, 1999, and it appeared that more than 2500 Belgian, French and Netherlands farms could have been supplied with contaminated feeds. Important features of the crisis were the creation of the Federal Agency for Food Safety in Belgium and the introduction in 1999 of norms for PCBs in feedstuffs and food in Belgium followed by the introduction in 2002 of European harmonized norms for PCDD/Fs in animal feed and food of animal origin. The Belgian crisis was followed few years later by an Irish pork PCB/dioxin contamination which source was traced to an animal feed production facility using the hot gases from the combustion of contaminated fuel oil to dry animal feed (Marnane, 2012).

Effects of feed contaminants on intestinal epithelium renewing and intestinal barrier function

Intestinal cell proliferation

The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals (Heath, 1996). The mammalian intestinal epithelium is renewed every 4–5 days. This high cell turnover allows rapid resealing of the epithelial barrier following injury or damage in order to maintain an effective barrier function. The normal homeostasis may be impaired by feed contaminants affecting self-renewing capacities of the

intestinal epithelium. The non-transformed porcine intestinal epithelial cell lines IPEC-1 and IPEC-J2 (see chapter Techniques for investigating gut function *in vivo*, *ex vivo* and *in vitro* in monogastric farm animals) have been used to assess mycotoxins toxicity on pig intestine (Bouhet *et al.*, 2004; Diesing *et al.*, 2011; Vandenbroucke *et al.*, 2011). Proliferating intestinal epithelial cells appears more sensitive to trichothecenes than differentiated cells, suggesting that exposure to these feed contaminants could dramatically impair the physiologically constant state of regeneration of the intestinal epithelium. Moreover, co-exposure to low concentrations of different trichothecenes leads to synergistic cytotoxicity in intestinal epithelial cells (Alassane-Kpembi *et al.*, 2013).

Intestinal Barrier function

Testing high concentrations of DON (2000ng/mL) on both intestinal cell lines resulted in disintegration of tight junction protein *zonula occludens-1* (ZO-1), increase of cell cycle phase G2/M and early activation of caspase-3, while low concentration (200ng/mL) showed no effect on these parameters (Diesing *et al.*, 2011). Reduced expression of other tight junction proteins, claudin 3 and claudin 4, was also reported in IPEC-1 monolayer epithelium exposed to DON (Pinton *et al.*, 2010; 2009). Other mycotoxins T2-toxin, FB1 and ZEA, also impair the integrity of IPEC-J2 monolayer via altered viability and reduced trans-epithelial electrical resistance (TEER), and promote the trans-epithelial passage of the antibiotics (Goossens *et al.*, 2012). It has also been observed that a prolonged exposure to FB1 prevents the establishment of the TEER and alters the resistance of an already established monolayer IPEC-1 epithelium (Bouhet *et al.*, 2004).

The mechanism by which mycotoxins could alter pig intestinal barrier function has also been investigated using intestinal cells and intestinal explants culture. The activation of the p44/42 ERK signaling pathway by DON and its acetyl derivatives inhibits the expression of tight junction claudin-4 protein, which leads to impaired

intestinal barrier function (Pinton *et al.*, 2012). Selective removal of claudin isoforms was also demonstrated with OTA which decreased intestinal barrier properties by repressing claudin 3 and 4, but not claudin 1 (McLaughlin *et al.*, 2004). Limited data related with chicken intestinal barrier function suggest that acute exposure to AFB1 moderately affect TEER (Yunus *et al.*, 2011b).

Beside the mycotoxins, PCBs constitute the other group of frequently occurring feed contaminants exerting an ascertained disruptive effect on intestinal epithelial integrity, even though no study to our knowledge assessed the specific sensitivity of farm animal models to this class of compounds. The disruptive effect of highly chlorinated PCBs on gut integrity has been demonstrated in human colon adenocarcinoma cells (Caco-2) and in C57BL/6 mice (Choi *et al.*, 2010). The authors showed that exposure to each of PCB congeners PCB153, PCB118, PCB104, and PCB126 increased the permeability of the intestinal barrier to fluorescein isothiocyanate (FITC)-labelled dextran (4 kDa) and disrupted expression of tight junction proteins ZO-1 and occludin.

Histo-morphological alterations of intestine induced by feed contaminants

A broad spectrum of histo-morphological alteration patterns is related to the presence of contaminants in feedstuffs. DON and its acetyl derivatives, FB1 and ZEA are associated with mucosal morphological abnormalities in poultry and pigs. Villi height decrease or atrophy as well as villi fusion and other regressive lesions in the gastrointestinal tract are frequently reported (Awad *et al.*, 2006; Kolf-Clauw *et al.*, 2009; Sklan *et al.*, 2003; Yunus *et al.*, 2012; Zielonka *et al.*, 2009). However hyperplasia of goblet cells was observed in 1-day-old broilers fed *ad libitum* for two weeks a 300 mg/Kg FB1-contaminated diet (Brown *et al.*, 1992). A chronic exposure of Ross 308 male chicks to increasing levels of AFB1, was associated with a dose dependent reduction of duodenum and jejunum weight (Yunus *et al.*, 2011a). Interestingly, a compensatory increase in the length of the duodenum and jejunum was noted after 4 weeks of exposure to high concentration of toxin. Contrary to the observations in

broilers, a linear increase in the crypts depth in distal jejunum was noted with increasing levels of AFB1 in the diet of layers (Applegate *et al.*, 2009).

Polychlorinated dibenzo-p-dioxins (PCDDs) are also associated with regressive intestinal lesions. A survey of environmental contamination with 2,3,7,8-TCDD and other PCDDs indicated that the intestine weight in two great blue heron (*Ardea herodias*) colonies regressed negatively on TCDD level (Sanderson *et al.*, 1994).

Modulation of digestive functionality of the intestine by feed contaminants

Absorptive functionality

The frequent regressive intestinal lesions may explain at least in part the reduced absorption of nutrients observed with exposure to several feed contaminants. Smith *et al.* (2012) reviewed evidence from human and animal studies that mycotoxins may share a downstream pathway for children stunting by targeting the intestinal tract and inducing Environmental Enteropathy. Aflatoxin reduced iron absorption in chicken, regardless of the presence of anemia (Lanza *et al.*, 1981). Surprisingly broiler chicks fed a 10 mg AFB1/Kg diet for one week showed an increase of the both mediated and passive components of glucose and methionine *in vitro* absorption, while lower exposure (1.25 to 5 mg) for longer period (3 week) showed no effect compared with control birds receiving no aflatoxin (Ruff and Wyatt, 1976).

Glucose uptake was assessed into laying hens jejuna epithelia in presence of DON (Awad *et al.*, 2007). DON decreased glucose uptake almost as efficiently as phlorizin. In the presence of phlorizin, pharmacological inhibitor of sodium glucose-linked transporter 1 (SGLT-1), DON had no additional effect on the glucose uptake. SGLT-1 is the main apical transporter for active glucose uptake in the small intestine. It works like a symporter that uses the electrochemical gradient of Na⁺ to drive the glucose absorption. This similarity between the effects of phlorizin and DON on glucose uptake evidences their common ability to inhibit Na⁺-D-glucose co-transport.

These authors also evaluated mRNA expression of SGLT-1 in broiler chicken fed a diet naturally contaminated with DON (1 and 5 mg/kg), (Awad *et al.*, 2011). After 5 weeks, the mRNA of SGLT-1 was down-regulated in duodenal and jejunal tissues of DON-supplemented groups. Ussing chamber experiments conducted at the same time confirmed the glucose-induced inhibition of currents in intestinal tissues. Taken together, these results suggest that gene expression mediate the inhibitory effects of DON on intestinal glucose uptake. Based on this transcriptomic approach, Dietrich *et al.* (2012) showed that not only DON impairs sugars (glucose and fructose) intestinal absorption in broilers, but that mycotoxin might also alter the uptake of palmitate and monocarboxylates in the jejunum at realistic doses of DON (2.5 to 5 mg/kg).

Conversely, sodium-dependent glucose absorption might be up-regulated in pig after acute or long term exposure to the mycotoxin FB1 (Lalles *et al.*, 2009; Lessard *et al.*, 2009). Studies in mice found that exposure to fumonisins *in utero* increases the frequency of developmental defects, and administration of folate or a complex sphingolipid is preventive (Marasas *et al.*, 2004). The folate uptake is mediated by the folate receptor, which like many glycosylphosphatidylinositol-anchored proteins, is enriched in cholesterol and sphingolipids. In the human intestinal epithelial Caco-2 model, the fumonisin induced-depletion in sphingolipids was shown to impair the folate receptor function (Stevens and Tang, 1997). The authors suggested that dietary exposure to FB1 could adversely affect folate uptake and potentially compromise cellular processes dependent on this vitamin. In pigs, sphingolipids depletion was observed in intestinal epithelium after a seven-days-dietary exposure to 1.5mg/kg b.w. FB1 (Loiseau *et al.*, 2007).

Active intestinal absorption of glucose and leucine may also be impaired after oral treatment with 2,3,7,8-TCDD or PCBs (Ball and Chhabra, 1981; Madge, 1976a; Madge, 1976b).

Activity of digestive enzymes

Modulation of digestive enzyme production and/or activity is one of the biological effects of food contaminants targeting the gastrointestinal tract. Several studies concluded that enzyme activity is modulated following mycotoxin consumption, even though contradictory effects were reported. In pigs the activity of aminopeptidase N, but not of sucrase, was markedly lowered by consumption of corn culture extracts containing fumonisins in a nine-day feeding trial (Lessard *et al.*, 2009). A 2-week feeding study with laying hens showed that the specific activity of the intestinal maltase increased quadratically by feeding up to 1.2 mg/kg of aflatoxin and declined at 2.5 mg/kg of aflatoxin (Applegate *et al.*, 2009). Activities of digestive enzymes from duodenum contents including protease, chymotrypsin, trypsin and amylase were increased in AFB₁-treated group for ducks in a six week- feeding trial (Han *et al.*, 2008). On the contrary α -amylase and lipase activities in duodenum were lower in hens fed the aflatoxin-contaminated diet while pancreas amylase, trypsin and chymotrypsin activity were increased (Matur *et al.*, 2010). In line with lipase activity decrease, there was a highly significant increase of lipid excretion in feces of young broiler chickens at 1.25 μ g/g and above dietary aflatoxin exposure (including a three-fold increase at 10 μ g/g) (Osborne and Hamilton, 1981). The paradoxical upward trend of pancreatic enzyme secretion might be ascribed to pancreas cells damage as acute and chronic pancreatitis are associated with great proenzyme release (Han *et al.*, 2008; Matur *et al.*, 2010). It's noteworthy that Osborne and Hamilton (1981) reported pancreatomegaly in chicken for 2.5 μ g/g dietary aflatoxin exposure and above.

The intestinal mucosa can also be regarded as the largest endocrine organ, which utilizes some 100 identified messengers (Ahlman and Nilsson, 2001; Furness *et al.*, 1999). The enterochromaffin cells constitute the largest endocrine cell population in the gastrointestinal tract. On their apical side, gut endocrine cells have microvilli, which may serve to taste the intraluminal milieu (Newson *et al.*, 1982). As an example cholecystokinin (CCK), also known as cholecystokinin-pancreozymin, is released

from duodenal endocrine cells after a meal. This hormone is produced by enteroendocrine I cells, which are distributed throughout the proximal intestine epithelium. It mediates digestive enzymes production from the pancreas and activates neurons of the gallbladder wall with subsequent emptying of bile for breakdown of fats and proteins in the meal. CCK also inhibits gastric emptying and initiates a satiety response via vagal afferents.

Chronic exposure to different types of commonly encountered environmental polychlorinated aromatic hydrocarbons (*i.e.* PCBs, dioxins, and 2,3,4,7,8-pentachlorodibenzofuran PeCDF) may exert an endocrine disruptive effect on intestinal CCK (Lee *et al.*, 2000). These authors demonstrated that chronic ingestion of PCB-126,2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or TCDD alone; a mixture of these three chemicals; or a mixture of PCB-126 and PCB-153 decreases intestinal stores of CCK peptide in a specific manner in rats. In addition, TCDD treatment of intestinal CCK cells lowered levels of prohormone convertase-1 and -2, which are involved in processing pro-CCK to mature, biologically active CCK.

Modification of the intestinal microflora by feed contaminant

As other fungi secondary metabolites especially antibiotics, several mycotoxins have shown their antimicrobial potential (Ali-Vehmas *et al.*, 1998; Burmeister and Hesseltine, 1966). As a consequence, mycotoxins may modify the intestinal microflora. In pigs trichothecenes DON and T-2 toxin modified the dynamics of the intestinal bacteria communities, causing an increase of the number of aerobic bacteria (Tenk *et al.*, 1982; Waché *et al.*, 2009). A significant decrease of the amount of *Salmonella* Typhimurium bacteria present in the cecum content, and a tendency to a reduced colonization of the jejunum, ileum, cecum, and colon contents was showed in presence of 15 and 83µg T-2 toxin per kg feed in pigs (Verbrughe *et al.*, 2012). Microarray analysis revealed that T-2 toxin causes an intoxication of *Salmonella* Typhimurium, represented by a reduced motility and a down-regulation of metabolic

and *Salmonella* Pathogenicity Island 1 genes. However, *in vitro* T-2 toxin promoted the susceptibility of intestinal epithelial cells to *Salmonella* Typhimurium invasion and its translocation over an intact intestinal porcine epithelial cell monolayer. The enhanced *Salmonella* invasion in and translocation over the intestinal epithelial IPEC-1 and IPEC-J2 cells was also observed with non-cytotoxic doses of DON (Pinton *et al.*, 2009; Vandebroucke *et al.*, 2011). Besides trichothecenes, the ability of OTA and patulin to increase the translocation of commensal bacteria across intact epithelium was also reported (Maresca *et al.*, 2008).

Effect of feed contaminant on secretion of some intestinal defence components

Mucus

In order to protect the mucosa, the host produces a complex layer of mucus that covers the gastrointestinal tract (Johansson *et al.*, 2011; McGuckin *et al.*, 2011). The mucus is organized in two layers that are organized around the highly glycosylated MUC2 mucin, forming a large, net-like polymer. The mucus gel provides a matrix for the retention of antimicrobial molecules in the mucosal environment. In addition, mucin glycoproteins that form the major macromolecular constituents of mucus can themselves have direct antimicrobial properties that limit the growth of microorganisms in the mucus. The mucin glycoproteins are produced by goblet cells. Feed contaminants may modulate mucus secretion.

ZEA and FB1 were found to exert a proliferative effect or an increasing activity on the goblets cells and their content of mucinogen vesicles in pigs and chickens (Brown *et al.*, 1992; Obremski *et al.*, 2005). On the contrary, low doses of mixtures of T-2 toxin, DON and ZEA reduced the number of goblet cells and the tightness of the intestinal glycocalyx in pigs (Obremski *et al.*, 2008).

Anti-microbial peptides

The other major secretory cells within the gastrointestinal tract are the Paneth cells, which are identified by their characteristic intracellular granules containing a range of antimicrobial molecules that are secreted into the mucus to ensure sterility of the stem cell niche. Among them are defensins, a family of cationic antimicrobial peptides containing a specific six-cysteine motif also produced by epithelial cells (Yang *et al.*, 2007). On porcine IPEC-J2 cells, an up-regulation of porcine beta-defensins 1 and 2 mRNA expression following exposure to DON, NIV, ZEA, individually and in mixtures has been observed (Wan *et al.*, 2013). Supernatants from IPEC-J2 cells exposed to toxins, singly or in combination, however, possessed significantly less antimicrobial activity against *Escherichia coli* than untreated supernatants. The results suggested interactive effects when cells were exposed to mycotoxin combinations.

Secretory IgA

Secretory antibodies, immunoglobulin A (IgA) and IgG that are very important components of the mucosal barrier, are secreted into the mucus by the epithelial cells (Strugnell and Wijburg, 2010). These antibodies influence the commensal microbiota and contribute substantially to the capacity of the mucus to retain and clear potential pathogens. Specific immunoglobulin (Ig) receptors are responsible for the immune response by regulating the Ig transport and cellular concentrations in various tissues. These receptors transport Igs across epithelial tissues to their sites of action. The Fc receptor (FcRn) is specific for IgG, whereas the polymeric immunoglobulin receptor (pIgR) recognizes dimeric IgA and pentameric IgM. Expression of the pIgR gene in epithelial cells of mucosal and glandular tissues is an unconditional pre-requisite for acquiring mucosal immunity, while FcRn could be important in immune activation and tolerance (Dickinson *et al.*, 1999; Verbeet *et al.*, 1995). In sheep, the silage contaminating mycotoxin mycophenolic acid (MPA) lowered FcRn expression in the liver, which may result in a lower IgG serum-to-bile transport, while pIgR expression

in ileum was stimulated (Dzidic *et al.*, 2004). For viruses that invade via the mucosal route, both IgA and IgG can provide protection and mediate viral clearance. In a mouse model, T-2 toxin impaired the gastrointestinal tract clearance of the enteric reovirus serotype 1 and increased its fecal shedding (Li *et al.*, 2006). These effects corresponded to a transient suppression of the induction of the specific IgA in feces and decreased secretion of reovirus-specific IgA and IgG_{2a} in Peyer's patch and lamina propria fragment cultures.

Also in mouse model, a single oral administration of low dose 2,3,7,8-TCDD resulted in a marked decrease in IgA secretion in the gut, showing that relatively low dose of dioxins may impair intestine mucosal immunity (Ishikawa, 2009; Kinoshita *et al.*, 2006). Altogether, these results bring strong evidence that feed contaminants may modulate the secretory antibody-dependant intestinal immune response.

Modulation of intestinal immune response by feed contaminants

When keeping the boundaries with the external world, the intestinal mucosa has to integrate internal and external signals for coordinating an innate and/or adaptative immune response (Maldonado-Contreras and McCormick, 2011). The deregulation of this intestinal mucosa immunomodulatory function may lead to intestinal inflammation or failure to face the continuous challenge of the transient and/or resident intestinal microflora. A finely tuned cross-talk between several cell lineages of the intestinal mucosa determines the homeostatic balance. This cross-talk is mediated by cytokines and chemokines which are small peptide molecules. Feed contaminants are able to modulate the production of these molecules. A heat map for the frequently regulated intestinal cytokines and chemokines in farm or experimental animals dietary exposed to DON, FB1, AF, T-2 toxin, OTA, alone or in combination has been proposed (Grenier and Applegate, 2013). A marked intestinal pro-inflammatory cytokines up-regulation, especially IL-6 and IL-8, is associated to mycotoxin exposure. The double-mechanism by which DON up-regulates the

chemokine IL-8 production is thought to involve the activation via MAPKinases and Protein Kinase R pathways of NF κ B-dependent transcription of the *IL-8* gene, and the HuR/ELAVL1 RNA binding protein-dependent stabilization of IL-8 mRNA (Pestka, 2010). Conversely, FB1 is known to decrease the basal expression and synthesis of IL-8 chemokine in pig intestine (Bouhet *et al.*, 2006). It makes sense that the subsequent reduction in inflammatory cell recruitment during intestinal infections explains at least in part the higher susceptibility of FB1 dietary exposed pigs (Oswald *et al.*, 2003). The same picture of down-regulation of the pro-inflammatory response can be observed in animal models exposed to TCDD or its congeners (Monteleone *et al.*, 2012; Monteleone *et al.*, 2011).

Impairment of the intestinal adaptative immunity is also attributed to exposure to feed contaminants. A decrease in intestinal IFN- γ production, the Th1 signature cytokine, is observed when trichothecene-exposed animals are orally challenged with enteric reovirus (Li *et al.*, 2005; Li *et al.*, 2006). As a consequence, those animals fail to clear the virus from their intestine.

Conclusion

Intestinal health is of great interest for all animals, especially monogastrics. Lengthy literature has been dedicated to the impact of chemicals on intestinal health. Data gathered show clearly that realistic doses of naturally occurring as well as anthropogenic feed contaminants impair intestinal functionality via different pathways. Physical barrier functionality of the gut, its digestive/absorptive role as well as its local defence mechanisms regulation assignment are sometimes simultaneously affected in numerous situations of dietary exposure to contaminants.

Moreover several contaminants may be present at the same time in the feed and interact together. More attention needs to be paid to the toxicological effect of contaminant mixture to determine whether they act additively, in synergy or

antagonism. There is a growing body of evidence pointing that mycotoxins may impair in a synergistic manner intestinal integrity (Grenier and Oswald 2012; Wan *et al.*, 2013; Alassane-Kpembé *et al.*, 2013). The characterization of these toxicological interactions deserves to be extended to other contaminant mixtures to improve our understanding of intestinal health risk associated with the presence of feed contaminants.

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2.2 Approches méthodologiques et pertinence biologique des études d'évaluation *in vitro* des interactions toxicodynamiques des mycotoxines

L'exposition aux mycotoxines constitue des cas d'école pour les études de toxicité des mélanges pour plusieurs raisons : (i) Un substrat alimentaire peut être colonisé simultanément par plusieurs moisissures productrices de différentes classes de mycotoxines ; (ii) une même souche de moisissure sécrète en général plusieurs mycotoxines, et (iii) les rations alimentaires sont constituées de divers aliments ayant une flore fongique et un profil de contamination mycotoxique qui leur sont propres. Identifier les interactions toxicodynamiques pour un mélange de composés revient à établir si les effets toxiques du mélange sont plus marqués, moins marqués ou conformes à un effet cumulatif théorique attendu des effets individuels des constituants du mélange. Pour un mélange de mycotoxines ou de tout autre type de xénobiotiques agissant sur un système biologique, une interaction toxicodynamique se traduit par un écart entre la toxicité observée, et la toxicité prédite sur la base de l'application d'un modèle d'additivité aux toxicités individuelles notées pour chacun des constituants du mélange. La caractérisation des relations dose-effet de chaque mycotoxine impliquée dans un mélange et de la relation dose-effet dudit mélange sont donc un pré-requis dans les études d'interaction. Cette conditionnalité est d'autant plus difficile à réaliser pour des études *in vivo* que le nombre de groupe expérimentaux, et donc d'animaux nécessaires, augmente drastiquement avec la complexité des mélanges étudiés. Les études *in vitro* apparaissent ici comme des alternatives intéressantes tout au moins pour les premières phases d'exploration des interactions toxicodynamiques.

Nous présentons dans cette revue une étude critique des différents concepts d'additivité pour les relations dose-effets et une synthèse des conclusions auxquelles elles ont permis d'aboutir pour les mélanges impliquant les principaux groupes de mycotoxines, et en utilisant diverses lignées de cellules humaines ou animales, transformées ou non transformées. Cette revue sera soumise pour publication au journal *Toxicology in vitro*.

Title: *In vitro* analysis of mycotoxin toxicological interactions: Methodological aspects and biological relevance.

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Abstract

Mycotoxins are secondary fungal metabolites produced under specific environmental conditions by a variety of mould spoiling agricultural commodities mainly *Aspergillus*, *Penicillium* and *Fusarium*. Human and animals are simultaneously exposed to several mycotoxins as evidenced by large-scale surveys. Simultaneous exposure to different toxins could result in synergistic effects. However, most toxicity studies addressed the effects of mycotoxins separately. Attempts are made by regulation agencies to set up new global recommendations. Here, we summarize the experimental designs and statistical aspects as well as the conclusions drawn from *in vitro* experiments exploring interactions in combined toxicity of mycotoxins

Introduction

Mycotoxins are secondary fungal metabolites produced under specific environmental conditions by a variety of mould spoiling agricultural commodities mainly *Aspergillus*, *Penicillium* and *Fusarium*. As secondary metabolites, they are not essential for life, but may provide the fungus with an ecological advantage in certain environments. Some 300 compounds have been recognized as mycotoxins of which a dozen is considered as threat to human and animal health. Mycotoxin exposure via food and feed may result in many different adverse health effects such as carcinogenicity, immunotoxicity, reproductive toxicity, hepatotoxicity, nephrotoxicity, *etc.* (Bennett and Klich, 2003). It has been estimated that at least 25% of the yearly worldwide grain production is contaminated (CAST, 2003).

Human and animals are simultaneously exposed to several mycotoxins as evidenced by large-scale surveys (Schothorst and van Egmond, 2004; Rodrigues and Naehrer, 2012). This is due to at least three different reasons (i) most fungi are able to simultaneously produce a number of mycotoxins, (ii) commodities can be contaminated by several fungi simultaneously or in quick succession and (iii) complete diet is made from various different commodities.

Because mycotoxins often co-occur in food stuffs, an increasing number of studies are devoted to their combined toxicity and attempts are made by regulation agencies to set up new global recommendations (SCF, 2002). Indeed, simultaneous exposure to different toxins could result in synergistic effects. Moreover, although the successful demonstration of synergism would justifiably heighten concerns about health risks, the possible implications of apparently less spectacular additive combination effects have not received adequate attention. In some cases the threshold dose for toxic effect may be exceeded for mixtures although the exposure to each single compound is considered as safe (Silva *et al.*, 2002).

Interactions are inferred when a mixture of chemicals produces a biological response greater or lesser than expected. Thus, the key question remains what is to be expected from a combination of agents. The application of the equations of Loewe additivity or Bliss independence criterion, basing on the dose–response curves of single compounds, enables the simulation of a theoretical response that represents for combination experiments, the expected behaviour of the mixture when interaction is excluded. Some reviews have been written about the subject and several aspects of the problem are still debated, with a particular regard to the biological plausibility of these two different theoretical approaches (Greco *et al.*, 1995; Chou, 2006; Goldoni and Johansson, 2007).

This review summarizes the experimental designs and statistical aspects as well as the conclusions drawn from *in vitro* experiments exploring interactions in combined toxicity of mycotoxins.

1. Experimental designs and statistical aspects in *in vitro* studies of mycotoxin toxicological interactions

Due to the dramatic increase in the number of experimental groups with increasing number of compounds in a mixture, most of *in vivo* mycotoxin combination studies focused on binary mixtures (Grenier and Oswald, 2011). *In vitro* bioassays are less restrictive in the number of test groups and therefore, make the hazard assessment of complex mycotoxin mixtures easier. As a consequence, several *in vitro* studies address more than two mycotoxins, or involve ternary combinations, and sometimes more complex mixture pattern. Classically, a two-step approach is recommended when analyzing mixture effects for possible pharmacological or toxicological interactions between the different compounds (Suhnel, 1996). First, one has to predict the combination effects expected for the case of no interaction, and in a second step the experimental combination effect data have to be compared to the expected ones in order to classify the combination as additive (no interaction *i.e.* as expected),

synergistic (*i.e.* interaction resulting in greater effect than expected) or antagonistic (*i.e.* interaction leading to lesser effect than expected). The definition of “no interaction case” means a clear statement of what effect size can be expected if the compounds in the mixture do not interact. Several experimental designs that denote authors’ point of view on this “no interaction case” have been used in *in vitro* assessment of mycotoxins combined effects.

In a number of studies, the expected mixture effect size was defined to equal the arithmetic sum of the sizes of individual compounds when tested separately (Weber *et al.*, 2005; Kouadio *et al.*, 2007; Ribeiro *et al.*, 2010; Ficheux *et al.*, 2012; Klaric *et al.*, 2012). As an example for this “no interaction case” the expected size for the cytotoxic effect of a mixture could be defined as sum of the cytotoxic effects induced by each mycotoxin alone in mono-exposition experiments, so:

$$\text{Cytotoxic effect (mycotoxin 1 + mycotoxin 2)} = \text{Cytotoxic effect (mycotoxin 1)} + \text{Cytotoxic effect (mycotoxin 2)}$$

Although intuitively plausible and very easy to handle, most researchers in the biomedical area seem to agree that combined effects do not simply equal the sum of single effects (Boedeker and Backhaus, 2010). Therefore this model of “no interaction case” to which many mycotoxin combined effects studies referred to does not provide a reasonable reference point.

Factorial designs experiments have been employed to assess interaction between mycotoxins *in vitro* (Tajima *et al.*, 2002a; Heussner *et al.*, 2006; Lei *et al.*, 2013; Wan *et al.*, 2013a; Wan *et al.*, 2013b). In that approach the “no interaction case” is defined as follow: when testing the effects of mixtures by different patterns of combination on the one hand and the effects of each individual compound on the other, the effect of any compound could be predicted by subtracting the mean of the groups not containing the compound from the mean of the other groups (Groten *et al.*, 1996). In a full factorial design, each of the chemical in the mixture is studied at all dose levels of

other chemicals, which may require a large number of tested groups and be very costly. Mycotoxins mixture studies favored the fractional factorials that enable more economy of experimentation because only part of the full factorial is run experimentally. As an example a three-step study was proposed to detect interactions between five *Fusarium* mycotoxins inhibiting DNA synthesis *in vitro*, which allowed to finally apply full factorial design to two-factor interactions of particular interest (Groten *et al.*, 1998). Despite the fact that interaction is definitely revealed by such statistical methods, the nature of interaction with regard to additivity, synergism or antagonism is not clearly explored and has to be inferred indirectly (Bhat and Ahangar, 2007). The approach could just point out “a potential for interactive (synergistic) effects of CIT and OTA and possibly other mycotoxins in cells of renal origin” (Heussner *et al.*, 2006).

The most commonly used theoretical biology-based models in the definition of zero interaction are the Bliss independent criterion also known as response addition, and the Loewe additivity model also named concentration or dose addition (Goldoni and Johansson, 2007). The main assumption in response addition is that the involved chemical agents act independently from one another. In other words the mode, and possibly also the site of action of the compounds in the mixture differs. When no interaction occurs for a combination, the response addition for two toxic agents can be expressed by the following equation:

$$E(x,y) = E(x) + E(y) - E(x)*E(y)$$

where E is the fractional effect (between 0 and 1), and x and y are the doses of two compounds in a combination experiment.

The concentration or dose addition model lies on the assumption that the toxic agents in the mixture of concern act on the same biological sites, by the same mechanisms of action and differ only in their potency. Some papers simultaneously tested both models of zero interaction for mycotoxins combined effects in *in vitro* experiments (Tammer

et al., 2007; Mueller *et al.*, 2013). Relatively simple Loewe additivity model extensions are the isobolographic method and its algebraic variant, the interaction index, particularly useful when assessing two toxic substances *in vitro*. The Interaction index I can be expressed as (Berenbaum, 1981):

$$I = c_1/EC_{x,1} + c_2/EC_{x,2}$$

with c_i denoting the applied concentrations (of agent 1 and 2, respectively) and EC_x their individual concentration that provokes a certain effect x ., e.g. the effect concentration 50% (EC_{50}). $I < 1$, $I > 1$ and $I = 1$ mean the agents interact synergistically, antagonistically, or are additive. This index has been applied in mycotoxin pairs association (McKean *et al.*, 2006a; McKean *et al.*, 2006b).

The isobolographic method may be considered the graphical variant of the Interaction index method (Tallarida, 2011). In a Cartesian coordinate system, doses of each agent that give a specified effect e.g. EC_{50} are represented on the x- and y-axes. The straight line connecting the equally effective doses of the agents is assumed to represent the set of dose pairs that give the specified effect in a situation of no interaction (additivity). Actual dose pairs that give the specified effect are then experimentally determined and reported on the graph. Experimental dose pairs lie below the additivity line in synergistic associations, and above in antagonistic associations. Isobolograms were drawn to analyze *in vitro* the interactions for *Penicillium* mycotoxins and *Fusarium* mycotoxins as well (Bernhoft *et al.*, 2004; Luongo *et al.*, 2006; Luongo *et al.*, 2008). However, the Interaction index method and the isobolographic method fail to take into account the variability of the data, and there is a need for further development of statistical methods for characterizing accurately the interaction of combination of agents (Gennings *et al.*, 1990). Furthermore, both methods strongly rely on the assumption of Loewe additivity *i.e.* the chemical agents of concern act on the same biological sites, by the same mechanisms of action, which is opposite to the Bliss independent criterion that holds for agents with strictly different mechanisms of action.

There is no final agreement on the biological plausibility of these concepts introduced for the analysis of combination effect and none of them found support by all researchers. There is a still ongoing debate on which is the “better” or even the “correct” concept (Boedeker and Backhaus, 2010). However, the Loewe additivity model is slightly preferred because of a general higher biological plausibility (Goldoni and Johansson, 2007). It is likely that most toxic substances exert actions that are not completely different and independent from those of other toxicants. In general the cellular damage is induced by binding of toxicants to macromolecules (proteins, DNA) or other molecules of significant biological activity and their inactivation induces consequent secondary processes, like oxidative stress, common to many toxicants. Moreover, because partially exclusive or partially non-exclusive cases may exist, it is possible to interpret effects as synergistic under assumption of Bliss independence, simply due to the fact that different toxic substances partially bind to the same target sites and do not have an independent action (Chou, 2006; Goldoni and Johansson, 2007).

Another concept that is independent of the mode of action and just considers both the potency (EC_{50}) and the shape of the dose-effect curve for each chemical agent and their mixture has been proposed (Chou, 2011). The new theory indicates that dose-effect relationships are not random or arbitrary events but rather governed by the physico-chemical principle of the Mass-action law, which allows computerized simulation of dose-effect curves and the “no interaction” response that could be expected from the combined effect of several agents (Chou, 2006). Thus this Mass-action Law-based deterministic approach is a distinct departure from traditional statistical methods for the dose-effect relationships analysis. Individual agents and their mixtures dose-effect relationship are biometrically modeled using the Median-Effect Equation of the Mass action Law that is:

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

where D is the dose of the agent (e.g. a cytotoxic mycotoxin), f_a is the fraction affected by D (e.g. percentage of viability 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).

Then interactions can be analyzed by a Combination-index-isobologram method derived from the Median-Effect Equation. This Combination index (CI) is calculated according to (Chou, 2011):

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

Where ${}^n(CI)_x$ is the combination index for n agents at $x\%$ inhibition, $(D)_j$ are the doses of n agents that exerts $x\%$ inhibition in combination, $(D_x)_j$ are the doses of each of n agents 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 agents. The calculation of this index allowed a quantitative assessment of interaction in a number of *in vitro* mycotoxin combined effect studies (Koshinsky and Khachatourians, 1992; Jones *et al.*, 1995; Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b; Kolf-Clauw *et al.*, 2013; Lu *et al.*, 2013). Another feature of the combination-index-isobologram method is that when synergy occurs in mixture effects, dose reduction indices (DRI) can be calculated. The DRI is a measure at a given effect level of how many folds the dose for an agent in a synergistic combination may be reduced compared with the dose of this agent when tested alone. For type B trichothecenes exhibiting low-dose synergistic cytotoxicity, this parameter showed its usefulness as correction factors that may take the observed synergies into account could be proposed (Alassane-Kpembi *et al.*, 2013).

2. In vitro combined toxicity studies involving ochratoxins

Ochratoxin A (OTA) has mainly been involved in studies addressing ochratoxins associations with other mycotoxins (Table 1 and Table 2). Very few papers mentioned ochratoxin B, the dechloro-analogue of OTA (Roth *et al.*, 1989; Heussner *et al.*, 2006). Ochratoxins are nephrotoxic compounds, and as a consequence, most of the *in vitro* studies involved human or animal renal cell lines or renal primary cells cultures. Cytotoxicity is the main endpoint explored for the mycotoxin combinations. Mycotoxins associations including ochratoxins have also been screened for genotoxicity via DNA damages, clastogenic effects and mutagenic activity. Mitogen-induced lymphocyte proliferation and cytokine production were the main endpoints for papers addressing ochratoxin combined toxicity in the immune system.

In vitro studies of the joint toxicity of ochratoxins and other Aspergillus and Penicillium mycotoxins

Ochratoxins are produced by several species in the genera *Aspergillus* and *Penicillium* and may co-occur with others mycotoxins produced by these species. Among these mycotoxins, citrinin (CIT) is the most frequently associated compound for combined toxicity studies (Table 1). Few papers also mentioned patulin (PAT), cyclopiazonic acid (CPA), gliotoxin, roquefortin (ROQ) and penicillic acid (PA) (Bernhoft *et al.*, 2004; Heussner *et al.*, 2006; Tammer *et al.*, 2007).

OTA and CIT have mostly been reported to act *in vitro* in a synergistic manner for their cytotoxic effects (Roth *et al.*, 1989; Bouslimi *et al.*, 2008a; Bouslimi *et al.*, 2008b; Klaric *et al.*, 2012). These mycotoxins were also considered synergistic for their genotoxic effects in liver-derived HepG2 cells (Knasmuller *et al.*, 2004). However, they failed to induce reverse mutation in the Ames test and showed antagonism in porcine kidney PK15 epithelial cells (Wurgler *et al.*, 1991; Klaric *et al.*, 2012). Considering endpoints for immunotoxicity, the synergy between OTA and CIT

was confirmed for mitogen-induced lymphocyte proliferation, while both mycotoxins were additive for the inhibition of IFN- γ production by peripheral blood mononuclear cells (Bernhoft *et al.*, 2004; Tammer *et al.*, 2007). Except CPA that showed antagonism in immunotoxicity, the combinations of OTA with other *Aspergillus* and *Penicillium* mycotoxins were reported as additive.

Table 1: Interactions between ochratoxins and other *Aspergillus* and *Penicillium* mycotoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Bernhoft <i>et al.</i> (2004)	OTA-CIT-CPA-PAT-ROQ-Pen Ac	Piglet Lymphocytes	Mitogen-induced lymphocyte proliferation	Dose-response curves IC20 isobologram	Synergy OTA-CIT, additivity OTA-CPA, Pen AC-RQ, PAT-RQ, PAT-Pen Ac, antagonism CIT-CPA
Bouslimi <i>et al.</i> (2008a)	OTA-CIT	Vero cells	Cell proliferation, DNA damage	Cytotoxicity comparison of IC ₅₀ values for individual toxins and their mixture, DNA damage comparison of the effects for several concentrations of individual toxins and their mixture	Synergy
Bouslimi <i>et al.</i> (2008b)	OTA-CIT	Vero cells	Cell proliferation, oxydative cell damage	Cytotoxicity comparison of IC ₅₀ values for individual toxins and their mixture, DNA damage comparison of the effects for several concentrations of individual toxins and their mixture	Synergy
Braunberg <i>et al.</i> (1994)	OTA-CIT	Pig renal cortical cubes	Protein synthesis, organic ions tetraethylammonium (TEA) and paraminohippurate (PAH) transport	Dose-response analysis for individual compounds and their mixture	Synergy and additivity for TEA and PAH ions transport and protein synthesis
Creppy <i>et al.</i> (1980)	OTA-CIT	Hepatoma tissue culture cells	Protein synthesis, RNA and DNA synthesis		
Heussner <i>et al.</i> (2006)	OTA OTB CIT PAT	Porcine renal cell line LLC-PK1	Cell viability	Step-wise approach (Full factorial+central composite designs)	Potential synergy OTA CIT

Table 1 continued.

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Klaric <i>et al.</i> (2012)	OTA-CIT	Porcine kidney epithelial cells PK15	Cell viability, apoptosis, necrosis, genotoxicity	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Additive effect for cell viability, synergy for apoptosis and necrosis, antagonism for genotoxicity
Knasmuller <i>et al.</i> (2004)	OTA-CIT	HepG2	Genotoxic effect, mitogenic effect		Possibly synergy
Knecht <i>et al.</i> (2005)	OTA-CIT	Human proximal tubule-derived cells (IHKE cells)	Cytotoxicity, apoptosis	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Antagonism for apoptosis at concentration of 2.5-5µM CIT, additivity at concentration 7.5-15µM
Pfohl-Leszkowicz <i>et al.</i> (2008)	OTA-CIT	Renal cell culture	Cytotoxicity, DNA adduct formation		
Roth <i>et al.</i> (1989)	OTA OTB - CIT	Hepatoma tissue culture cells	Protein synthesis	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Slight synergy
Tammer <i>et al.</i> (2007)	OTA-CIT-PAT-GLIO	Peripheral blood mononuclear cells	Cell viability, cytokine production	Comparison of actual mixture toxicity data to predicted ones based on concentration addition and response addition concepts	Additivity
Wurgler <i>et al.</i> (1991)	OTA-CIT	<i>Salmonella</i> Typhimurium strains TA 102	Mutagenic activity		No reverse mutation

In vitro studies of the joint toxicity of ochratoxins and Fusarium mycotoxins

Publications related to the *in vitro* combined toxicity of ochratoxins and *Fusarium* mycotoxins group concern Fumonisin B1 (FB1), and in a lesser extent the emerging mycotoxin beauvericin (BEA) (Table 2). Conflicting conclusions have been proposed for the interactive cytotoxic effects of OTA and FB1. The combination studies resulted in synergistic effects (Creppy *et al.*, 2004; Carratu *et al.*, 2005; Mwanza *et al.*, 2009) and in additive response as well (Klaric *et al.*, 2007; Klaric *et al.*, 2008a). Binary and ternary combinations for OTA, FB1 and BEA mainly depicted an additive genotoxic potential for the mixtures.

Table 2: Interactions between ochratoxins and *Fusarium* mycotoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Carratu <i>et al.</i> (2005)	OTA-FB1	Caco-2	Protein synthesis inhibition	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Synergy
Chiarappa <i>et al.</i> (2004)	OTA-FB1	Caco-2, C6, Vero cells			
Creppy <i>et al.</i> (2004)	OTA-FB1	Vero cells, caco-2 cells, C6 glioma cells	Cell viability	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Synergy
Klaric <i>et al.</i> (2007)	OTA-BEA-FB1	Porcine kidney epithelial cells PK15	Cell viability, lipid peroxydation (TBARS) and GSH depletion	Combination of equal concentrations of two or all three mycotoxins	Additivity, possibly synergy and antagonism
Klaric <i>et al.</i> (2008a)	OTA-BEA-FB1	Porcine kidney epithelial cells PK15	Cell viability, apoptosis	Comparison of the toxic effects of individual mycotoxin and binary and ternary mixture of equal concentrations of the toxins	Additivity for cell viability, additivity and synergy for apoptosis
Klaric <i>et al.</i> (2008b)	OTA-FB1	Porcine kidney epithelial cells PK15	Clastogenic effect	Combination of equal concentrations of two or all three mycotoxins	Additivity for presence of Micro Nuclei and for presence of Nucleoplasmic Bridges

Table 2 continued.

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Klaric <i>et al.</i> (2010)	OTA BEA	Porcine kidney epithelial cells PK15, Human leukocytes	Genotoxic potential	Combination of two concentrations	Additivity and synergy in PK15, additivity in HL
Mwanza <i>et al.</i> (2009)	OTA-FB1	Pig lymphocytes, Human lymphocytes	Cell viability	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Synergy
Stoev <i>et al.</i> (2009)	OTA-FB1-CIT	Human peripheral blood mononuclear cells	Mitogen-induced cell proliferation MTT	Comparison of the toxic effect of the mixture to each of the toxic effects of individual compounds at their concentration in the mixture	Stronger effect of the mixture compared to any individual compound

3. In vitro studies of combined toxicity involving aflatoxins

In this review publications related to mycotoxin mixtures involving aflatoxins have been grouped in (i) studies presenting aflatoxin-aflatoxin combined effects (table 3) (ii) studies presenting the combined effects of aflatoxin B₁ and other *Aspergillus* or *Penicillium* mycotoxins (Table 4) and (iii) studies presenting the joint toxicity of aflatoxin B₁ and *Fusarium* mycotoxins (Table 5).

Aflatoxins are hepatocarcinogenic agents in numerous animal species and have been implicated in the etiology of human hepatocellular carcinoma (Wild and Montesano, 2009). In association with other mycotoxins, the mutagenic effects and cell viability of aflatoxins have been frequently questioned. Nearly all the papers addressing mutagenic activity of these mycotoxin combinations referred to the well known Ames test using *Salmonella* Typhimurium strains TA 100 and TA 98. However a bioluminescence test using the marine bacterium *Photobacterium phosphoreum* Strain NCMB 844 was also proposed (Yates *et al.*, 1987). Mutagenicity was analyzed with a dark mutant of this organism which reversion to the bioluminescent condition is stimulated by compounds attacking guanine sites in desoxyribonucleic acids. Aflatoxins combinations have been assessed for their cytotoxic and genotoxic effects mainly in human and animal primary hepatocytes or transformed cell lines (Friedman *et al.*, 1997; He *et al.*, 2010; Ribeiro *et al.*, 2010; Corcuera *et al.*, 2011). Aflatoxin combinations are considered potentially immunotoxic, thus they have been evaluated for their combined effects on the viability and functionality of immune system cells (Theumer *et al.*, 2003; Herzog-Soares and Freire, 2004; Russo *et al.*, 2010; Theumer *et al.*, 2010; Russo *et al.*, 2011).

In vitro studies of the joint toxicity of aflatoxins

The aflatoxins are a group of closely related highly substituted coumarins containing a fused dihydrofurofuran moiety. Four aflatoxins may occur naturally: the two blue

fluorescent toxins (B₁, B₂) that are characterized by fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety, and the two greenish yellow fluorescent toxins (G₁, and G₂) contain a fused lactone ring. AFB₂ and AFG₂ are considered relatively nontoxic unless they are first metabolically oxidized to AFB₁ and AFG₁ *in vivo*. The metabolism of aflatoxin B₁ and B₂ in the mammalian body may result in two metabolites M₁ and M₂ as hydroxylated derivatives of the parent compound. Concentration ratios of 1.0 to 0.1 for AFB₁ and AFB₂ and 1.0:0.1:0.3:0.03 for AFB₁, AFB₂, AFG₁, and AFG₂ proportions have been reported when aflatoxins co-occur in diets (Kensler *et al.*, 2011). However, these ratios can be variable. Cell viability as an endpoint showed synergy for the combined toxicity of AFB₁ and AFB₂ in human umbilical vein endothelial cells (HUVEC) while additivity was observed for the same endpoint in human lung fibroblast (HFL) and in human ovarian cancer cell line A 2780 (Braicu *et al.*, 2010). No interaction was also reported between AFB₁ and AFB₂ for RNA synthesis and membrane integrity in rat hepatocytes primary culture, while an undetermined interaction was revealed between AFB₁ and AFG₁ (Friedman *et al.*, 1997). The immunotoxic interactions between aflatoxin metabolites excreted in milk on the one hand and between these metabolites and the parent-compounds on the other have been investigated (Russo *et al.*, 2010; Russo *et al.*, 2011; Bianco *et al.*, 2012b). No interaction could be detected when macrophages were co-exposed to AFM₁ and AFM₂, while their combinations with the parent-compounds AFB₁ and AFB₂ resulted in stronger toxicity compared to individual toxins, which suggested synergism to the authors.

Table 3: Interactions between aflatoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Bianco <i>et al.</i> (2012b)	AFB1-B2-M1-M2	J774A.1 murine macrophages	Cell viability, apoptosis, inhibition of nitric oxide production	Comparison of IC ₃₀ values for viability reduction for individual toxins and mixtures, comparison of nitric oxide production inhibition by graded levels of individual toxins and mixtures	stronger effects of mixtures compared to individual toxins suggesting interactions
Braicu <i>et al.</i> (2010)	AFB1 AFB2 AFG1 AFG2	Human umbilical vein endothelial cells (HUVEC), human lung fibroblasts (HFL), and A2780	Cell viability	Comparison of IC ₅₀ values for mixtures and individual toxins	synergy AFB1-AFB2 in HUVEC cells, additivity (AFB1-AFB2-AFG1-AFG2) in HFL and A2780
Friedman <i>et al.</i> (1997)	AFB1 AFB2 AFG1	Rat hepatocytes, rat liver slices	RNA synthesis, membrane integrity	Two-way ANOVA	No interaction AFB1-AFB2, indetermined interaction AFB1-AFG1
Russo <i>et al.</i> (2010)	AFM1 AFM2	J7741.A murine macrophages	Cell viability, activation of macrophagic functions (Nitric oxid production)	Comparison of the effects of serial dilutions of individual toxins and their combination	No interaction
Russo <i>et al.</i> (2011)	AFB1, AFB2, AFM1, AFM2	J7741.A murine macrophages	Cell viability, activation of macrophagic functions (Nitric oxid production)	Comparison of the effects of serial dilutions of individual toxins and their combination	Stronger effects of mixtures compared to individual toxins suggesting interactions

In vitro combined toxicity of aflatoxin B1 and other *Aspergillus* or *Penicillium* mycotoxins

OTA and CPA are the two mycotoxins produced by *Aspergillus* and/or *Penicillium* strains that have been frequently associated to AFB₁ for mixture toxicity studies (Table 4). A number of *Aspergillus* strains that produce B- and G-type aflatoxins may also produce CPA (Lee and Hagler, 1991; Pildain *et al.*, 2008). As a consequence CPA and aflatoxins often co-contaminate crops (Urano *et al.*, 1992; Chang *et al.*, 2009). The ratio of aflatoxins:CPA co-occurring in maize ranged from 16.6:1 to 1.6:1 (Gqaleni *et al.*, 1996). OTA has been demonstrated to be carcinogenic among laboratory animals, and rated as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC). This may justify its frequent association to the carcinogenic AFB₁ in *in vitro* joint toxicity studies. Conflicting conclusions for the clastogenic effect of AFB₁-OTA association exist. A quantitative analysis of DNA fragmentation in cultured monkey kidney Vero cells exposed to both mycotoxins simultaneously, suggested an additive effect (Golli-Bennour *et al.*, 2010). On the contrary, OTA was shown to reduce the DNA damages caused by AFB₁ alone in the transformed hepatocyte cell line HepG2 (Corcuera *et al.*, 2011). However, AFB₁ may have an increase of its mutagenic effect in presence of OTA (Sedmikova *et al.*, 2001).

Following metabolic activation by either human S-9 mix or rat S-9 mix, the mutagenic activity of AFB₁ and CPA combination assessed by reverse mutation of *Salmonella* Typhimurium TA 98 and TA 100 strains constantly resulted in a reduction compared to AFB₁ individual effect (Kuilman-Wahls *et al.*, 2002; Vilar *et al.*, 2003). On the opposite, the marine bacterium *Photobacterium phosphoreum* reverse mutation test revealed an enhanced genotoxic effect for AFB₁ in mixture with CPA (Yates *et al.*, 1987).

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Table 4: Interactions between Aflatoxin B₁ and other *Aspergillus* and *Penicillium* mycotoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Corcuera <i>et al.</i> (2011)	AFB1 OTA	HepG2	Cytotoxicity and genotoxicity	Comparison of IC ₅₀ values for mixtures and individual toxins	Additive effect for cytotoxicity, antagonism for genotoxicity
Golli-Bennour <i>et al.</i> (2010)	AFB1 OTA	Vero cells	Cytotoxicity and genotoxicity	Determination of the IC ₅₀ values for individual toxins and mixture, and calculation of their Interaction index	Additivity
Herzog-Soares <i>et al.</i> (2004)	AFB1 CIT	Mice macrophage	Infectivity and proliferation of <i>Toxoplasma gondii</i>	Comparison of infectivity and proliferation in control, individual and combined toxin groups	Increased infectivity and proliferation of <i>T. gondii</i> for the combined exposure compared to control
Kuilman-Wahls <i>et al.</i> (2002)	AFB1 Cyclopiazonic acid (CPA)	<i>Salmonella</i> Typhimurium strains TA 100 and TA 98	Mutagenic activity following metabolic activation by rat S-9 mix	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture	Reduction of AFB1 mutagenic activity
Sedmikova <i>et al.</i> (2001)	AFB1 OTA	<i>Salmonella</i> Typhimurium strains TA 100 and TA 98	Mutagenic activity	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture	Significant increase of the mutagenic activity of AFB1
Vilar <i>et al.</i> (2003)	AFB1 Cyclopiazonic acid (CPA)	<i>Salmonella</i> Typhimurium strains TA 100 and TA 98	Mutagenic activity following metabolic activation by human S-9 mix	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture	Reduction of AFB1 mutagenic activity

Table 4 continued

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Yates <i>et al.</i> (1987)	AFB1 Cyclopiazonic acid	Marine bacterium <i>Photobacterium phosphoreum</i> strain NCMB 844 and strain NRRLB 1177	Genotoxicity and cytotoxicity	Comparison of the effects for individual toxin doses and dose pairs in mixture	Enhanced genotoxic effect of AFB1 by CPA

In vitro combined toxicity of aflatoxin B₁ and Fusarium mycotoxins

The simultaneous spoilage of food commodities by *Aspergillus* and *Fusarium* strains is not uncommon and may be associated to natural co-occurrence of aflatoxins and various *Fusarium* mycotoxins, including DON, NIV, ZEA and FB1 (Ali *et al.*, 1998; Almeida *et al.*, 2012). Data on the combined toxicity of aflatoxins and these fusariotoxins are presented in Table 5.

The IARC has classified FB1 as possibly carcinogenic to humans (group 2B). The co-occurrence of the two carcinogenic mycotoxins AFB₁ and FB₁ was observed in 100% of the aflatoxin-contaminated Brazilian corn samples (82 samples) (Vargas *et al.*, 2001). No enhancement of the clastogenic effect has been noted when combining FB1 and AFB₁ in rat primary hepatocyte and spleen mononuclear cell culture, whereas biomarkers of oxidative stress were lowered by the mixture compared to the individual AFB₁ effect (Ribeiro *et al.*, 2010; Theumer *et al.*, 2010). The mutagenic activity of AFB₁ was surprisingly significantly enhanced by trichothecene mycotoxins DON and T-2 toxin which demonstrated no individual effect by their own (Smerak *et al.*, 2001). Related to immunobiological effects, the mixture AFB₁- FB₁ was more effective in reducing the mitogenic response and cytokine production of mononuclear cells on the one hand and H₂O₂ release of adherent peritoneal cells on the other, compared to the individual mycotoxins (Theumer *et al.*, 2003). Concerning the cell viability as an endpoint, synergy in toxicity was noted between AFB₁ and T-2 toxin or DON or ZEA and antagonism between AFB₁ and FB₁ (McKean *et al.*, 2006a; McKean *et al.*, 2006b; Lei *et al.*, 2013). It is noteworthy that the type of toxic interaction in cell viability between AFB₁ and *Fusarium* toxins, especially trichothecenes, may depend on the cell model as additivity was reported in fish primary hepatocytes and human hepatoma cells (HepG2) while synergy was observed in human bronchial epithelial cells (BEAS-2B) (McKean *et al.*, 2006b; He *et al.*, 2010).

Table 5: Interaction between aflatoxin B₁ and *Fusarium* mycotoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
He et al. (2010)	AFB1 DON	<i>Cyprinus carpio</i> primary hepatocytes	Cell viability (MTT test), enzyme (Aspartate aminotransférase AST, Alanine transférase ALT, Lactate dehydrogenase LDH) activity in cell supernatant	Comparison of the effects of the mixture and the effects of individual toxins One-way ANOVA	Additivity
Lei et al. (2013)	AFB1-FB1-ZEA-DON	Porcine Kidney 15 (PK15) cells	Cell viability (MTT), membrane damage (LDH), apoptosis and oxidative stress	Factorial design	Synergistic cytotoxicity for AFB1-ZEA, AFB1-DON, low dose antagonism and high dose synergy ZEA-AFB1 synergy DON-AFB1 for oxidative damage
McKean et al. (2006a)	AFB1 FB1	Human hepatoma cells (HepG2), Human bronchial epithelial cells (BEAS-2B)	Cell viability	Dose-response curves determination of IC50 values for individual toxins and their mixture, Calculation of interaction index metric	Additivity BEAS-2B, antagonism HepG2
McKean et al. (2006b)	AFB1 T-2	Human hepatoma cells (HepG2), Human bronchial epithelial cells (BEAS-2B)	Cell viability	Dose-response curves determination of IC50 values for individual toxins and their mixture, Calculation of interaction index metric	Synergy in BEAS-2B, additivity in HepG2
Ribeiro et al. (2010)	AFB1 FB1	Rat primary hepatocytes culture	Cell viability, DNA fragmentation and apoptosis	Comparison of the toxic effects of individual mycotoxin and mixture doses	No toxicity enhancement

Table 5 continued.

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Smerak et al. (2001)	AFB1 T-2 toxin DON	Prokaryote model (<i>Salmonella</i> Typhimurium, strains TA98 and TA100)	Mutagenic activity	Comparison of the effects of individual toxins and their mixtures	Significant enhancement of the mutagenic effect of AFB1 (no activity for T2 and DON alone, but greater activity for the combinations with AFB1)
Theumer et al. (2003)	AFB1-FB1	Rat Wistar spleen mononuclear cells (SMC) and adherent peritoneal cells (APC)	Mitogenic response and cytokines (IL-2, IL-4, IL-10) production of SMC and H ₂ O ₂ release of APC	Comparison of the effects of 20 µmol/l AFB1, to the effects of a mixture 20 µmol/l AFB1 + 10 µmol/l FB1	Differences in the effects produced by a mixture of mycotoxins in comparison to the individual action of the same toxins
Theumer et al. (2010)	AFB1-FB1	Rat Wistar spleen mononuclear cells (SMC)	Genotoxicity (alkaline comet assay and micronuclei assay) and oxidative stress (malondialdehyde (MDA) levels, catalase (CAT) and superoxide dismutase (SOD) activities)	Comparison of the individual effects of 20 µg/ml FB1 and 10 µg/ml AFB1 to the effects of a mixture of 20 µg/ml FB1 + 10 µg/ml AFB1	No difference in DNA injury, no difference in MDA levels, higher CAT and SOD activities in AFB1 individual treatment compared to FB1, and the mixture

4. Interactions between *Fusarium* mycotoxins

In this review, publications related to the in vitro assessment of *Fusarium* mycotoxins are grouped in articles exploring the combined effects of trichothecene mycotoxins (Table 6) and articles presenting the joint toxicity of the other fusariotoxins with or without trichothecenes (Table 7). Not all the trichothecenes involved in association studies are *Fusarium* mycotoxins. The non-macrocylic trichothecenes produced by *Fusarium* species have been combined to the macrocylic trichothecenes roridin A and verrucarol A produced by *Myrothecium* species (Koshinsky and Khachatourians, 1992; Jones *et al.*, 1995). For most papers, the mycotoxin association patterns involved the widely known *Fusarium* toxins trichothecenes, FB1 and/or ZEA, although increasing attention is being paid to combinations including the emerging mycotoxins BEA and ENN (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b; Flicheux *et al.*, 2012; Kolf-Clauw *et al.*, 2013; Lu *et al.*, 2013). Joint toxicity studies mainly investigated the cytotoxicity or immunotoxicity of *Fusarium* mycotoxins. Due to its oestrogenic activity, mixtures including ZEA have also been tested on the reproductive system cells (Malekinejad *et al.*, 2007; Ranzenigo *et al.*, 2008).

In vitro combined toxicity of trichothecenes

The type and intensity of interactions depicted between trichothecenes may vary accordingly with the tested doses and the combination ratios (Table 6). Synergistic cytotoxicity and immunotoxicity limited to the low doses were detected combining DON, NIV and their acetyl derivatives, while the interaction between type A trichothecenes and roridin A changed from antagonistic to synergistic for toxicity levels towards the yeast *Kluyveromyces marxianus* (Koshinsky and Khachatourians, 1992; Severino *et al.*, 2006; Alassane-Kpembi *et al.*, 2013). Combination of the type B trichothecenes DON or NIV to the type A T-2 toxin or DAS resulted in additive or antagonistic response either for the cytotoxic or the immunotoxic endpoints

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(Thompson and Wannemacher, 1986; Thuvander *et al.*, 1999; Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b).

Table 6: Interaction between trichothecenes

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Alassane-Kpembi et al. (2013)	DON - 3-ADON - 15-ADON - NIV - FX	Caco-2	Cytotoxicity NR MTT	CI-Isobologram method	Low-dose synergies, antagonism for ternary mixture DON-NIV-FX
Bianco et al. (2012a)	DON-NIV	Rat intestinal epithelial cell line IEC-6	Cell viability, apoptosis, cell migration	Incubation with graded levels of DON or NIV alone or in combination	No additive or synergistic effects
Jones et al. (1995)	T-2 toxin Verrucaridin A	Yeast <i>Kluyveromyces marxianus</i>	Growth inhibition	Calculation of Combination index value for binary mixtures at various ratios	The type and intensity of interactions varied with the combination ratios, growth percent inhibition and the growth medium: In a rich medium synergy over a 2-3 log value concentration range for a 1.0 µg/ml T-2 toxin:0.75 µg/ml verrucaridin A ratio
Koshinsky and Khachatourians (1992)	HT-2 toxin T-2 toxin Roridin A	Yeast <i>Kluyveromyces marxianus</i>	Growth inhibition	Calculation of Combination index value for binary mixtures at various ratios	The type of interaction varied accordingly to the mixture ratios and the percent of inhibition of growth: from antagonism to synergy for increasing inhibition of yeast growth
Marzocco et al. (2009)	DON NIV	J7741.A murine macrophages	Cell viability, Pro-apoptotic activity	Comparison of the IC ₅₀ values of the toxins and their mixture	No synergy
Severino et al. (2006)	DON-NIV	Jurkat T cells	Lymphocyte proliferation and cytokines expression	Incubation with graded levels of DON or NIV alone or in combination ratio 1:1 and 10:1 for DON:NIV	Interactive effect for lymphocyte proliferation, and interactive effects at lower concentrations (0.06-4µM) for IFNγ and IL2 mRNA transcription
Thompson and Wannemacher (1986)	12-13 epoxytrichothecenes	Vero cells, rat spleen lymphocytes	Protein synthesis inhibition	Comparison of IC ₅₀ values of different ratios binary mixtures to the IC ₅₀ values of the individual toxins	Additivity
Thuvander et al. (1999)	T-2 toxin DON NIV DAS	Human lymphocytes	Mitogen-induced lymphocyte proliferation Cell viability, Immunoglobulin production	Comparison of the mixture toxicity and the individual toxicity of 2× the concentration of toxin used at the combined exposure	Additivity (NIV - T2, NIV - DAS, NIV - DON), and slight antagonism (DON - T2, DON - DAS) for lymphocytes proliferation

In vitro combined toxicity of trichothecenes, FB1, ZEA and the “emerging” Fusarium mycotoxins

Most joint toxicity studies are related to simultaneous contamination by type B trichothecenes (DON and or NIV), FB1 and ZEA or its alcohol metabolite α -zearalenol (Table 7). The combination between the main *Fusarium* mycotoxins was shown to act additively on the porcine Ipec J2 cell viability reduction while interaction occurred for pro-inflammatory cytokines mRNA expression and the modulation of the expression of β -defensins 1 and 2 (Wan *et al.*, 2013a; Wan *et al.*, 2013b; Wan *et al.*, 2013c). Their toxicity was also found additive for the inhibition of DNA synthesis in mouse fibroblast (Groten *et al.*, 1998; Tajima *et al.*, 2002a). In binary association, synergy was reported between ZEA or its alcohol metabolite α -zearalenol and FB1 for various endpoints and cell systems (Groten *et al.*, 1998; Tajima *et al.*, 2002a; Luongo *et al.*, 2006; Kouadio *et al.*, 2007; Luongo *et al.*, 2008). The synergy may also exist for the combined anti-proliferative effect of ZEA and DON on porcine granulosa cell but it was not confirmed for other endpoints in pig reproductive toxicology (Malekinejad *et al.*, 2007; Ranzenigo *et al.*, 2008).

The emerging *Fusarium* toxins beauvericin and enniatins have been involved in mycotoxins combined effects studies for their cytotoxic and genotoxic potential. Binary and ternary mixtures of ENN A, A1, B and B1 exerted synergistic cytotoxicity on ovarian cells and intestinal cells (Lu *et al.*, 2013; Prosperini *et al.*, 2013). On the contrary, the toxicity of T-2 toxin was down-modulated by ENN B1 in pig intestinal epithelial cells and explants culture (Kolf-Clauw *et al.*, 2013). No interaction could be detected in combined myelotoxicity for ENN B and BEA, while synergy was shown in BEA and DON mixture (Ficheux *et al.*, 2012). In other cell lines antagonism was rather observed for the latter association (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b). This cell line-related discrepancy was also noted for the combined toxicity of BEA and the type A trichothecene T-2 toxin (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b). The

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combination of BEA to FB1 led to an additive induction of apoptosis in mononuclear cells (Dombrink-Kurtzman, 2003)

Table 7: Interactions between *Fusarium* mycotoxins

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Boeira et al. (2000)	DON, ZEA, FB1	Brewing yeast strains (Saccharomyces cerevisiae, Kluyveromyces marxianus, Pichia membranaefaciens, Hansenula anomala, and Schizosaccharomyces pombe)	Yeast growth	Comparison of the growth inhibition for combinations and the sum of the inhibition for toxins alone	interaction at high concentration: synergism or antagonism depending on toxin combination ratios
Bouaziz et al. (2012)	T-2 Zea	Vero cells	Cytotoxicity MTT ROS production, and expression of Heat shock protein HSP70	Combination of toxins at equimolar concentration	Increased toxicity compared to each toxin alone
Dombrink-Kurtzmann (2003)	FB1-BEA	turkey peripheral blood mononuclear cells	Apoptosis assessed by nuclear DNA fragmentation	8µM FB1, 8µM BEA, 8+8µM FB1 &BEA	slightly additive effect
Ficheux et al. (2012)	DON-BEA-T2-Zea-ENN	Human Colony Forming Unit-Granulocyte and Macrophage, CFU-GM	myelotoxicity	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	synergy DON-BEA, antagonism DON-FB1, synergy or additivity DON-T2, additivity DON-ZEA, T2-ZEA, BEA-ENN B,

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Table 7 continued

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Font <i>et al.</i> (2009)	BEA DON T-2	CHO-K1 (chinese hamster ovary cells), Vero cells	Cell viability NR	Comparison of the tested toxicity of mycotoxin mixtures and predicted toxicity (simple additive)	Potential of interactive effects
Groten <i>et al.</i> (1998)	T-2 toxin DON NIV ZEA FB1	L929 cells	DNA synthesis inhibition	Factorial design	Mainly additive combinations and synergy for ZEA-FB1, NIV-T2
kolf-clauw <i>et al.</i> (2013)	ENN B1 -T2	Ipec 1 and intestinal explants	cytotoxicity and morphological and histopathological scoring	Isobologram and CI methods	Less than additivity with decreasing concentrations of toxins
Kouadio <i>et al.</i> (2007)	DON ZEA FB1	Human intestinal cell line Caco-2	Malonedialdehyde (MDA) production, DNA and protein synthesis inhibition, DNA methylation, DNA fragmentation, cell viability, lipid peroxidation	comparison of the effects of Binary and tertiary mixtures to the sum of the effects of each toxin alone	Antagonism FB1-ZEA for cell viability, synergy ZEA-FB1 and ZEA-DON for lipid peroxydation, far less than additivite in DNA synthesis inhibition for binary mixtures of DON, FB1 and ZEA
Lu <i>et al.</i> (2013)	ENN A, A1, B & B1	Hamster ovarian cells CHO-K1	Cytotoxicity MTT	CI-Isobologram method	Synergistic effect of combined ENs A+A1, A+B, A1+B1, A+A1+B, A+A1+B1, A+B+B1 and A1+B+B1
Luongo <i>et al.</i> (2006)	FB1 a- zearalenol	Jurkat T cells	Lymphocyte proliferation, cytokine (IL-2 and INF-gamma) expression	Dose-response curves and isobologram	Synergy for lymphocyte proliferation, interactive effect cytokine expression

Table 7 continued

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Luongo <i>et al.</i> (2008)	FB1 DON NIV a-zearalenol	Swine whole-blood culture	Cell proliferation	Dose-response curves and isobologram	Synergy (FB1 a-Zea), no interaction (DON NIV)
Malekinejad <i>et al.</i> (2007)	DON ZEA	Pig oocytes	Abnormalities in formation of the meiotic spindle, Inhibition of oocyte maturation, developmental competence of matured oocytes after in vitro fertilization	Comparison of the effects of various ratios leading to 3.12µM/L mixture DON-ZEA to the effects of 3.12µM/L of each toxin alone of DON ZEA mixture	No synergy
Prosperini <i>et al.</i> (2013)	ENN A, A1, B & B1	Caco-2 cells	Cytotoxicity MTT	CI-Isobologram method	Synergy for ENN B – ENN A1, ENN B1 – ENN A1, ENN A – ENN A1 – ENN B ; antagonism for ENN B – ENN B1; additivity for all other combinations
Ranzenigo <i>et al.</i> (2008)	DON ZEA	Porcine granulosa cells	Granulosa cell proliferation, steroidogenesis, gene expression	Comparison of the effects of 30 ng/mL of DON, 30 ng/mL of ZEA and both	Possibly synergy for alteration of GC proliferation
Rimando <i>et al.</i> (1999)	Fusaric acid + DON & FA + FB1	Pineal cell cultures	Levels of pineal 5HT and 5HTP	1µM FA, 1µM DON, 1µM FB1, 1+1µM DON&FA, 1+1µM FA+FB1	Possibly synergy or antagonism
Ruiz <i>et al.</i> (2011a)	DON-BEA-T2	Hamster ovarian cells CHO-K1	Cytotoxicity MTT and NR	CI-Isobologram method	DON-BEA antagonism, DON-T2 antagonism, BEA-T2 synergism, DON-BEA-T2 synergism and low dose antagonism

Table 7 continued

Reference	Mycotoxin association	Cell model	Endpoint	Study design	Combined effect
Ruiz <i>et al.</i> (2011b)	DON-BEA-T2	Vero cells	Cytotoxicity NR	CI-Isobologram method	DON-BEA antagonism, T2-BEA antagonism, DON-T2 antagonism, DON-BEA-T2 antagonism
Severino <i>et al.</i> (2008)	DON-NIV-ZEA-FB1	Human Jurkat cells and porcine lymphocytes	Mitogen-activated lymphocyte proliferation	Increasing concentrations of each mycotoxin with constant concentrations of others	Interaction
Tajima <i>et al.</i> (2002b)	T-2 toxin DON NIV ZEA FB1	L929 cells	DNA synthesis inhibition	Factorial design	Mainly additive combinations and synergy for ZEA-FB1, NIV-T2
Wan <i>et al.</i> (2013a)	DON-NIV-ZEA-FB1	Ipec J-2	Cell viability	Factorial design	Non-additive interactions except DON-FB1-ZEA
Wan <i>et al.</i> (2013b)	DON-NIV-ZEA-FB1	Ipec J-2	Pro-inflammatory cytokines IL1 α , IL1 β , IL6, IL8, TNF α and MCP-1 mRNA expression	Factorial design	Non-additive interactions
Wan <i>et al.</i> (2013c)	DON-NIV-ZEA-FB1	Ipec J-2	Modulation of the expression of beta-defensin 1 & 2	ANOVA	Non-additive interactions

Conclusion

The present review analyzed the methodological aspects and main conclusions drawn from *in vitro* studies of mycotoxins toxicological interactions. Several approaches have been used for the definition of the additive effect that serves as a reference point in interaction analysis. Which of these approaches is better or even correct is still debated. Compared to *in vivo* studies, *in vitro* studies are less restrictive in the number of tested groups, so several cases of analysis of interaction involving more than two mycotoxins are reported. A number of *in vitro* models with different metabolic abilities along with various mycotoxin association patterns have been used, that may explain substantial discrepancy in the mycotoxin interaction study conclusions.

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II. TRAVAIL EXPERIMENTAL

OBJECTIFS DE LA THESE

L'intestin représente la première barrière de l'organisme contre les contaminants alimentaires. Elle joue un rôle protecteur, mais constitue également une cible pour ces contaminants, au nombre desquels les mycotoxines tiennent une place importante. Le DON se classe parmi les mycotoxines les plus fréquemment rencontrées dans les aliments partout dans le monde. Comme en attestent également les enquêtes, la probabilité d'une exposition du consommateur à une seule mycotoxine est très faible, et la co-contamination des aliments est la règle plutôt que l'exception. Le DON est ainsi retrouvé avec d'autres TCTs B, le 3-ADON, le 15-ADON, le NIV et la FX.

Au niveau cellulaire, les TCTs inhibent la synthèse protéique en se fixant à la sous-unité 60S du ribosome. Ils exercent donc une toxicité plus marquée au niveau des processus biologiques nécessitant un renouvellement cellulaire important. Les TCTs B présents dans les aliments pourraient ainsi impacter le renouvellement de l'épithélium intestinal nécessaire entre autres à la fonction de barrière de l'intestin, et à la digestion et l'absorption des nutriments.

A l'image d'autres inhibiteurs de la synthèse protéique (ricine, anisomycine et shigatoxine) dont le ribosome est la cible, les TCTs induisent une "Réponse au Stress Ribotoxique" (RSR) aboutissant entre autres choses à la cascade des MAPkinases. Ces voies de la signalisation cellulaire sont associées à certains mécanismes effecteurs de l'immunité, notamment l'inflammation. En conséquence, les TCTs B présents dans les aliments pourraient altérer l'homéostasie de la réponse immunitaire intestinale face aux sollicitations antigéniques importantes provenant à la fois de la flore aussi bien saprophyte que pathogène, et des constituants alimentaires.

La première partie de la thèse avait pour objectif d'étudier l'effet inhibiteur des TCTs B pour le renouvellement de l'épithélium intestinal, (i) en caractérisant et en comparant le pouvoir inhibiteur exercé par cinq TCTs B, le DON et ses dérivés acétylés 3-ADON et 15-ADON, et le NIV et son dérivé FX, et (ii) en évaluant et en

quantifiant les interactions toxicodynamiques en termes de synergie, additivité ou antagonisme, qui pourraient résulter d'une exposition simultanée de l'intestin à ces TCTs B.

Quant à la deuxième partie de la thèse, elle avait pour objectif d'évaluer la sensibilité de la réponse pro-inflammatoire au niveau intestinal, en tant que marqueur de la toxicité des TCTs B en caractérisant les relations dose-effet de ces mycotoxines par rapport à l'activation d'un certain nombre de gènes impliqués dans cette réponse. Ce travail constitue également la première étape de l'étude des interactions dans l'activité pro-inflammatoire combinée des TCTs B vis-à-vis de l'intestin. Les expérimentations et les analyses de ces interactions sont en cours, et ne seront pas présentées dans cette thèse.

CHAPITRE I :

TOXICITE COMPAREE ET COMBINEE DES TRICHOTHECENES B POUR LE
RENOUVELLEMENT DE L'EPITHELIUM INTESTINAL

Les résultats de ce travail sont présentés sous forme d'articles :

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F.A., Oswald, I.P., Puel, O., 2013. New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. *Toxicol Appl Pharmacol* **272**, 191-198.

Alassane-Kpembi I., Oswald I.P., and Puel O. Toxicological interactions between deoxynivalenol, nivalenol and their acetyl derivatives in intestinal epithelial cells. *Soumis pour publication à Toxicology*.

RESUME DE L'ETUDE

Dans l'organisme, les tissus et organes ayant un grand pouvoir de régénération sont décrits comme particulièrement sensibles à la toxicité des trichothécènes qui sont avant tout des inhibiteurs de la synthèse protéique. Pour le renouvellement de l'épithélium intestinal, nous avons voulu dans cette première partie du travail expérimental, (i) caractériser et comparer le pouvoir inhibiteur exercé par cinq TCT B, le DON et ses dérivés acétylés 3-ADON et 15-ADON, et le NIV et son dérivé FX, et (ii) évaluer et quantifier les interactions toxicodynamiques en termes de synergie, additivité ou antagonisme, qui pourraient résulter d'une exposition simultanée de l'intestin à ces TCTs B.

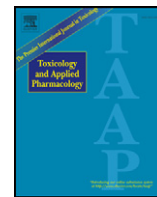
En utilisant des modèles humain et porcine d'entérocytes en croissance nous avons montré que les cellules épithéliales intestinales en croissance sont sensibles aux TCTs B testés, avec des IC_{50} de l'ordre du micromolaire, la toxicité de la FX étant la plus marquée ($IC_{50} = 0,04 \mu M$ sur lignée humaine). Dans les deux lignées cellulaires, les mycotoxines peuvent être classées par sensibilité croissante 3-ADON, DON, 15-ADON, NIV et F-X, mais des différences de toxicité relative des TCTB et de leurs dérivés acétylés entre lignée porcine et lignée humaine ont été observées.

L'analyse des interactions a été faite par la méthode de Chou et Talalay. Pour les mélanges synergiques, une quantification de l'amplitude de synergie a été faite sur la base des réductions de doses observées pour chaque composé par rapport à la dose reflétant l'additivité. Nous avons ainsi pu montrer que le type d'interactions entre TCT B varie en fonction des doses, avec notamment des synergies aux faibles doses. Pour les concentrations en mycotoxines équivalentes aux niveaux d'exposition rapportés pour les consommateurs européens, les amplitudes d'interaction observées indiquent que les synergies correspondaient à des réductions de dose d'un facteur compris entre 3 et 10.



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



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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.

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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-acetyldeoxynivalenol) 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 world. 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; IC₅₀, inhibitory concentration 50%; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; NIV, nivalenol.

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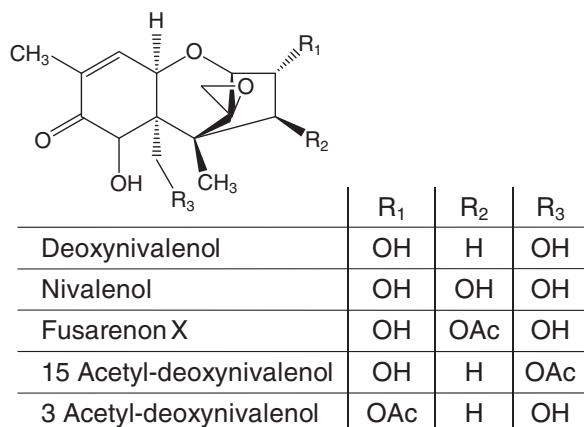


Fig. 1. Chemical structures of DON and NIV and their acetyl derivatives.

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-cm² 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% CO₂ 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. IC₅₀), 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

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

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 \pm 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 μ M. 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 μ M (Table 1).

For the overall ranking, mycotoxins were cytotoxic for proliferating enterocytes in the increasing order 3-ADON < 15-ADON \approx DON < NIV \ll 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 derivatives was further analyzed by calculating CI value at various cytotoxicity levels. The CI/f_a curves for binary and ternary

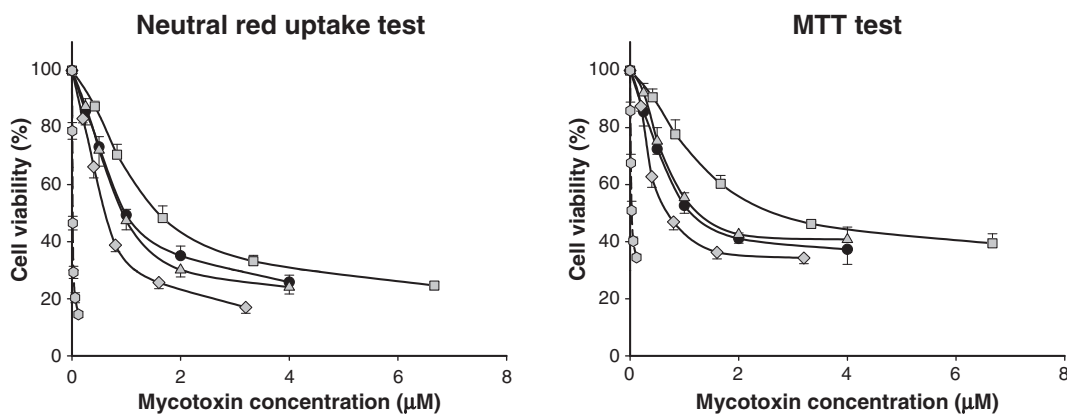


Fig. 2. Comparative cytotoxicity of Type B trichothecenes on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of DON (○), 3-ADON (□), 15-ADON (△), NIV (◇) or FX (○). Cytotoxicity was assessed by the MTT and neutral red assays. Data are means \pm SD of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

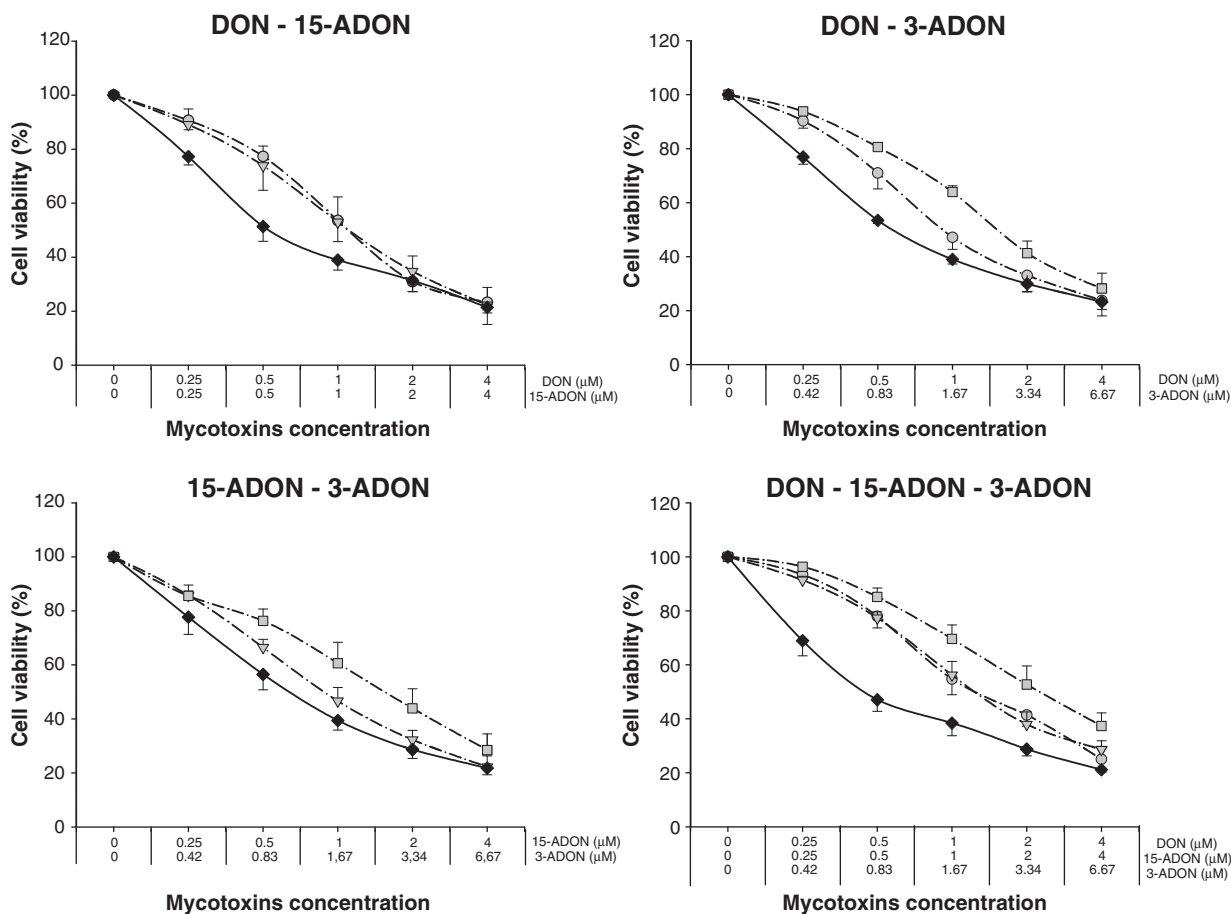


Fig. 3. Comparative toxicity of DON (○), 3-ADON (◻) and 15-ADON (▽) alone or in binary or ternary mixtures/combinations (◆) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral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

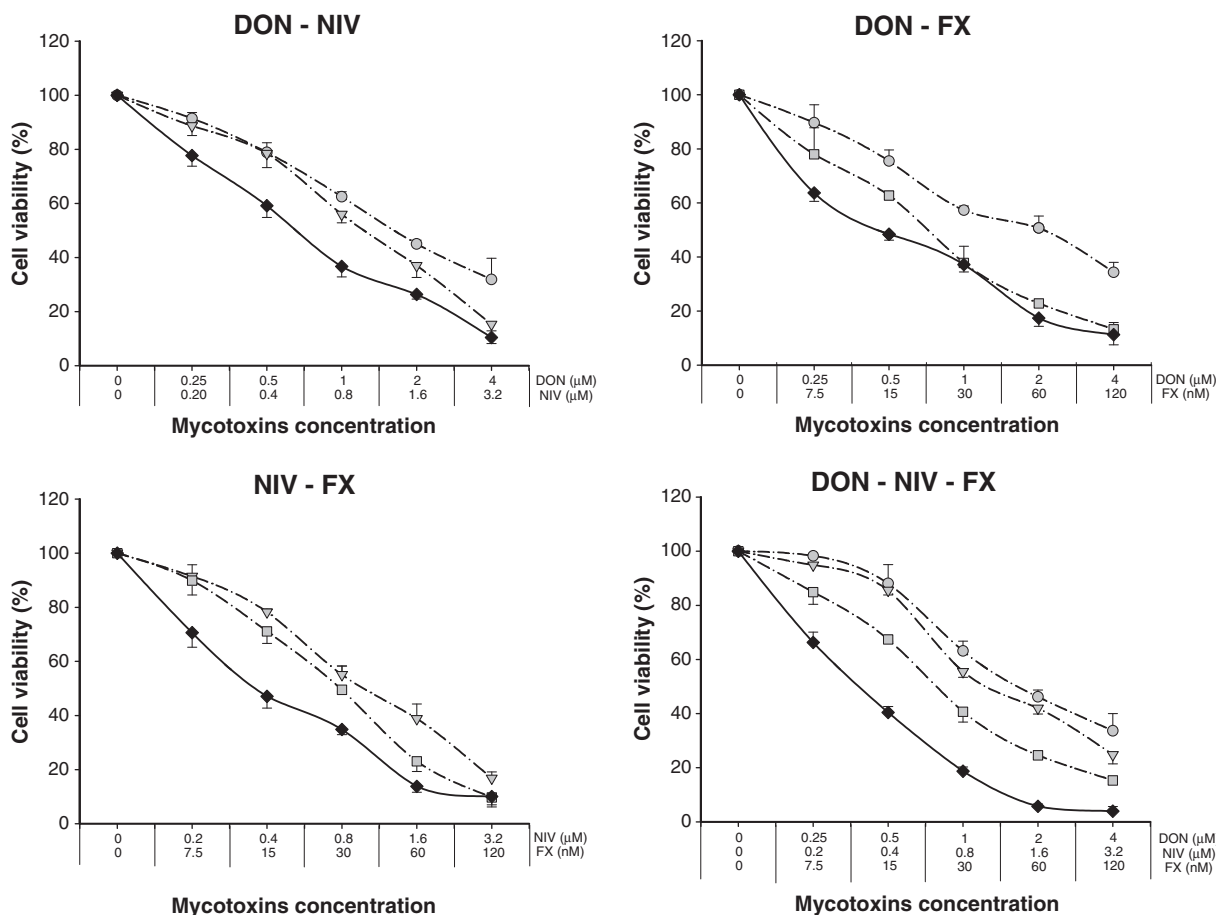


Fig. 4. Comparative toxicity of DON (○), NIV (▽) and FX (◻) alone or in binary or ternary mixtures/combinations (◆) on proliferating Caco-2 cells. Intestinal epithelial cells were exposed for 48 h to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the neutral red assay. 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 IC₅₀ and the relative toxicity of the five selected mycotoxins were similar with both cytotoxicity tests.

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 IC₅₀ 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.,

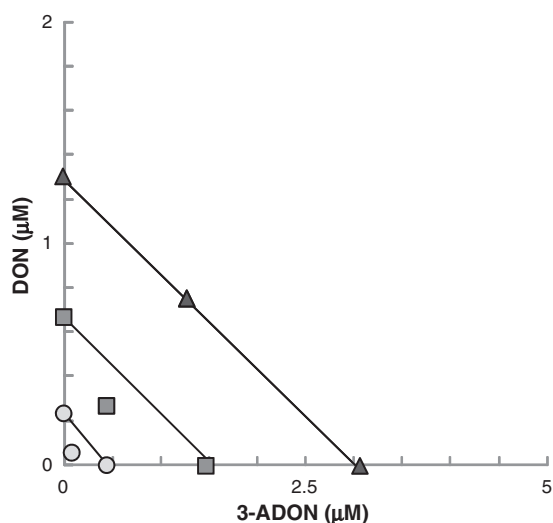


Fig. 5. Isobologram illustrating the combined cytotoxicity of DON and 3-ADON. At concentrations eliciting 10% (○), 30% (◻) or 50% (▲) toxicity. The points are mean concentrations of dose–response neutral red cytotoxicity curves for each toxin or toxin combination (Compusyn software analysis).

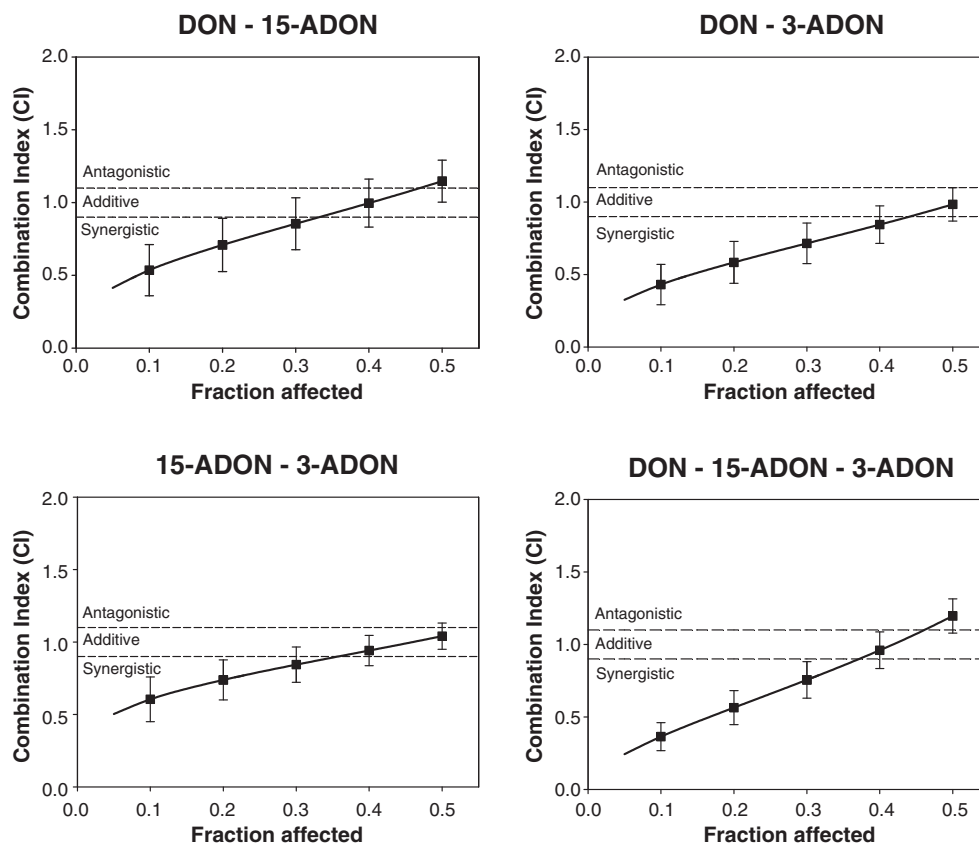


Fig. 6. Combination index – fraction affected curve for binary and ternary combinations of DON, 3-ADON and 15-ADON. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for 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			1.9		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 IC₁₀ and IC₃₀, 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

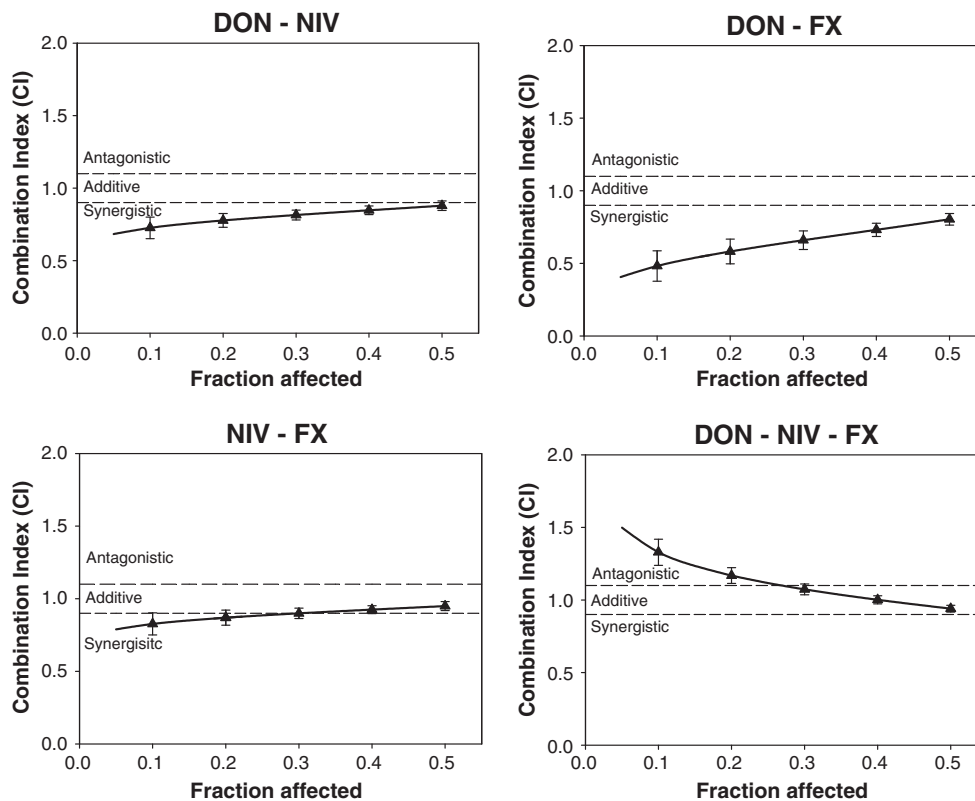


Fig. 7. Combination index – fraction affected curve for binary and ternary combinations of DON, NIV and FX. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. The vertical bars indicate 95% confidence intervals for CI values 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 widely 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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Title: Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetyl derivatives in intestinal epithelial cells

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Running title: Toxicological interactions between trichothecenes

List of relevant abbreviations and definitions:

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

IC₅₀: Inhibitory concentration 50 %

MTT: 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide

NIV: nivalenol

Abstract

Food and Feedstuff is usually contaminated by more than one mycotoxin, however toxicological data concerning the effects of mycotoxin combinations are lacking. 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 (TCT B), 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 tested mycotoxins and their binary mixtures had a dose-dependent effect on the proliferating enterocytes, with IC_{50} values for single compounds ranging from 2 to 20 μ M. The interactions between DON and NIV; DON and 15-ADON; 3-ADON-15-ADON were synergistic. Additivity was observed between NIV and FX, and antagonism between DON and FX. The association between DON and 3-ADON demonstrate an antagonism at low doses and a synergy at higher doses. 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 TCT B in the diet.

Introduction

Trichothecenes are fungal secondary metabolites produced by genera including *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma* and *Trichothecium* (McCormick *et al.*, 2011). They represent a unique class of mycotoxins that 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 (Proctor *et al.*, 1995; McLean, 1996; 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 (Brasel *et al.*, 2004; Pestka *et al.*, 2008; McCormick *et al.*, 2011). The adverse health effects of trichothecenes include emesis, nausea, anorexia, growth retardation, hemorrhagic lesions, neuroendocrine changes and immunosuppression (Pestka, 2010; Maresca 2012). At the molecular level, trichothecenes display multiple inhibitory effects on primary metabolism of eukaryotic cells including inhibition of proteins as well as DNA and RNA synthesis, and their activity may eventually produce harmful levels of oxidative stress due to generation of free radical (Suneja *et al.*, 1989; Rocha *et al.*, 2005). 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 first barrier against food contaminants. It 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 (Heath, 1996; Maresca *et al.*, 2008).

Type B trichothecenes (TCT B) are exclusively produced by *Fusarium* species. Due to a high prevalence of *Fusarium* head blight caused by *F. graminearum* and *F. culmorum*, TCT B 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) (Fig 1) were present in 57%, 20%, 16%, 10% and 8%, respectively of food samples collected in the European Union (Schothorst and van Egmond, 2004). *Fusarium* species spoiling food and feed matrices may also produce several mycotoxins simultaneously, especially DON, NIV and their acetyl derivatives (Sugiura *et al.*, 1990). Several studies have shown these trichothecenes to co-occur in raw materials, food and feed (Kim *et al.*, 1993; Eckard *et al.*, 2011; Schollenberger *et al.*, 2012, Streit *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; Grenier and Oswald, 2012). Multi-exposure may lead to additive, synergistic or antagonist toxic effects. The aim of the present study was to assess the combined effects of five type B trichothecenes in terms of additive, antagonistic or synergistic toxicity towards intestine epithelial cells. IPEC-1 cell line was chosen for the current study because it is a better model of normal small intestinal epithelial cell line than colon-derived transformed cell lines such as Caco-2 to study toxin interactions with intestinal mucosa (Brosnahan and Brown, 2012). It was anticipated that the results obtained in the present study would provide a better understanding of possible effects of TCT B, alone or in combination on human or animal health risks.

2. Materials and methods

2.1. Toxins

DON, 3-ADON and 15-ADON were purchased from Sigma (St Quentin Fallavier, France). They were dissolved in DMSO to 15 mM, except 3-ADON that was dissolved to 40 mM. NIV and FX 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.

2.2. Cell culture and cytotoxicity assay

IPEC-1 cells, derived from the small intestine of a newborn unsuckled piglet were maintained as previously described (Bouhet *et al.*, 2004). 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 CellTiter 96® Non-Radioactive MTT assay (Promega, Charbonnières-les-Bains, France) was run according to the protocol described by the manufacturer. The assay is based on the cellular conversion of a tetrazolium salt (MTT) into a formazan product by mitochondrial succinate dehydrogenase. In brief, 15 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) was added per well before a 4-hour incubation at 37°C in 5% CO_2 . One hundred μL of a solubilization/stop mixture were then added, and after an additional 1-hour room temperature incubation, the optical density (OD) was read at 570 nm using an Infinite M200 multiplate reader (TECAN, Werfen, Austria). IPEC-1 cells seeded in 96-well plates, were incubated 24-hour at 39°C , before being treated for 24 hours with serial dilutions of single or combination of mycotoxins or vehicle (DMSO) alone. The final toxin concentrations tested ranged from $0.12\mu\text{M}$ to $150\mu\text{M}$. 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.

The percentage of viable cells was calculated using the formula

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

2.3. Data analysis

Median-Effet and Combination Index-Isobologram analysis of mycotoxin mixtures.

Dose-effect studies for single mycotoxins, and binary associations were carried out simultaneously. The dose-effect relationships 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 than 0.95 (Chou, 2010).

Mycotoxin interactions were analyzed by the isobologram and combination-index methods as previously described (Alassane-Kpembi *et al.* 2013). This later index was calculated according to Chou (2011).

$${}^n(\text{CI})_x = \sum_{j=1}^n \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^n \frac{(D_x)_{1-n} \{ [D]_j / \sum_1^n [D] \}}{(D_m)_j \{ (f_a)_x / [1 - (f_a)_j] \}^{1/m_j}}$$

Where ${}^n(\text{CI})_x$ 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, $([D]_j / \sum_1^n [D])$ is the proportionality of the dose of each of n toxins that exerts $x\%$ inhibition in combination, and $(D_m)_j \{ (f_a)_x / [1 - (f_a)_j] \}^{1/m_j}$ is the doses of each of n toxins alone that exerts $x\%$ inhibition.

Regardless of the mechanisms or the units of the drugs $CI = 0.9-1$, $CI < 0.9$, 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, the dose reduction index (DRI) was calculated. 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. DRI can be obtained by setting the reciprocal of the CI equation:

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

and

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

Statistical analysis

The reported values are the means \pm 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.

The dose-effect relationship analysis for individual mycotoxin cytotoxicity, 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).

3. Results

3. 1. Individual and combined cytotoxicity of type B trichothecenes on Ipec-1 cells

We evaluated simultaneously the cytotoxic effects of individual and combined type B trichothecenes on porcine intestinal epithelial cells. The mycotoxins and their mixtures clearly showed a dose-dependent toxicity toward proliferating Ipec-1 cells (Fig 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 viability inhibition 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 ($r > 0.95$), which qualifies for further analysis using the Median-Effect principle. m values are slopes that depicted the shape of the dose-effect curve for individual and combined mycotoxin cytotoxicity experiments. D_m is the effective concentration for a 50% inhibition of cell proliferation. The D_m values for individual toxins DON, 15-ADON, NIV and FX were in the same range, while 3-ADON has roughly a ten-fold higher D_m which means it was ten-fold less potent. Combining the D_m and m values for the single mycotoxins and their mixtures, the analysis of the types of interaction can be done by drawing isobolograms, and calculating CI values. By means of that Combination Index-Isobolgram method, we evaluated the nature of the interactions for the combined toxicity of DON, NIV and their acetyl derivatives on proliferating porcine intestinal epithelial cells.

3. 2. Synergistic associations of type B trichothecenes

A typical isobologram is presented in Fig 3 for the combination of DON and 15-ADON at three different cytotoxicity levels (10%, 30% and 50%). 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. In the same figure, we can observe the synergistic effect of DON and 15-ADON at the investigated cytotoxicity levels. For further analysis we calculated the CI values for a wide range of cytotoxicity levels.

For binary mixtures of DON, NIV and acetyl derivatives, the evolution of CI values for graded levels of cytotoxicity is presented in Fig 4. Along the graded cytotoxicity levels, the DON – 15-ADON binary combination mainly demonstrated synergy, with the higher limit of the 95% confidence interval for the CI values lower than 0.9. A similar pattern of this synergistic activity was also noted for the 3-ADON – 15-ADON binary combination. 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 decreased from 7 at lower toxicity level to 2 at higher toxicity level, indicating that the lower the toxicity level, the higher the synergy magnitude. Conversely, DON – NIV mixture depicted a constant-magnitude synergy that was less intensive (Fig 4, Table 2).

3. 3. 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 4). 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. 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).

4. Discussion

Toxins from *Fusarium* sp. are very common contaminants of food and feedstuffs (Abouzied *et al.*, 1991; Streit *et al.*, 2012). Mycotoxin contamination of cereal grain commodities, collected in the course of national monitoring programs in Northern Europe during a 20-year period, revealed that DON had the highest overall incidence (Van Der Fels-Klerx *et al.*, 2012). During the last years, the shift from single analyte methods to multi-target methods has given a better picture of mycotoxin contamination (Berthiller *et al.*, 2007; Hajslova *et al.*, 2011; Capriotti *et al.*, 2012; Streit *et al.*, 2013). There is growing evidence that co-contamination of food matrices is the rule, not the exception. DON is often present with other TCT B such as 3-ADON, 15-ADON, NIV and FX (Kim *et al.*, 1993; Eckard *et al.*, 2011; Schollenberger *et al.*, 2012). The present study was designed to assess the combined toxicity of TCT B.

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 (Rocha *et al.*, 2005). Organs and/or tissues showing high rate of cell turnover are particularly susceptible to trichothecenes. The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals (Heath, 1996). 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 junction proteins (Pinton *et al.*, 2010; Pinton *et al.*, 2012; Bracarense *et al.*, 2012). One outcome of this work was to determine how DON, NIV and their

acetyl derivatives could jointly impair intestinal barrier function by affecting epithelial cell renewing.

Considering the similarities in digestive physiology, pig may be considered an illustrative case of dual-purpose domestic animal models that benefit agricultural and biomedical research, especially for the mycotoxin health risk assessment issues (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 (Loiseau *et al.*, 2007; Pinton *et al.*, 2010; Goossens *et al.*, 2012). 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 (Pinton *et al.*, 2012; Brosnahan and Brown, 2012). Non-differentiated pig enterocytes appeared clearly very sensitive to DON toxicity (Vandenbroucke *et al.*, 2011), suggesting that exposure to this contaminant could dramatically impair the physiologically constant state of regeneration of the intestinal epithelium. In this work, TCT B and their mixtures clearly showed a dose-dependent toxicity toward proliferating IPEC-1 cells. The D_m value or IC_{50} reported here for DON is in accordance with previous cytotoxicity studies using the IPEC-1 model (Danicke *et al.*, 2010; Diesing *et al.*, 2011b). 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 cell line (Wan *et al.*, 2013) as well as in the rat intestinal epithelial cell line IEC-6 (Bianco *et al.*, 2012). In the present study, we observed a similar toxicity of NIV and FX (4-acetyl NIV) suggesting that the presence of an acetyl group does 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) (Pinton *et al.*, 2012). This is in line with the known structure-activity relationship of trichothecenes (Ikawa *et al.*, 1985; Thompson and Wannemacher, 1986; Desjardins *et al.*, 2007). 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). By contrast, a higher toxicity of FX compared to NIV was noted in the human colorectal carcinoma cell line Caco-2 (Bony *et al.*, 2007; Alassane-Kpembi *et al.*, 2013).

The combined toxicity of *Fusarium* toxins DON and NIV on IPEC J-2 cells has been previously investigated (Wan *et al.*, 2013). Using a factorial design approach, the authors concluded to a non-additive interaction for the joint reduction of cell viability by DON and NIV, though the type of interaction in terms of synergy or antagonism was not specified. Interaction if any may be revealed by factorial experiments, but such statistical methods fail to reveal the exact nature of interaction with regard to synergism or antagonism (Bhat and Ahangar, 2007). In the present study we used the Chou-Talalay method to analyze the interaction. This method combines a qualitative assessment of interactions via an isobolographic analysis and a quantification of synergy or antagonism by combination and dose reduction indexes at different toxicity levels (Jones *et al.*, 1995; Ruiz *et al.*, 2011; Kolf-Clauw *et al.*, 2013; Lu *et al.*, 2013). We could thus characterize the non-additive interaction for DON and NIV as a synergy with a constant magnitude whatever the toxicity level. Synergistic interaction between DON and NIV has already been reported in mouse fibroblast L-929 cells (Tajima *et al.*, 2002), and in a yeast bioassay (Madhyastha *et al.*, 1994) as well as in Caco-2 cells (Alassane-Kpembi *et al.*, 2013). In the present experiment, the analysis of the interactions was extended to other TCT B. A synergy was observed not only between DON and NIV, but also between DON and 15-ADON, between 3-ADON and 15-ADON and at high toxicity level between DON and 3-ADON. The magnitude of the synergy was determined by calculating the DRI. This index represents the ratio between the observed and predicted doses of TCT in mixtures to reach the same toxicity. In the present experiments the DRI values ranged from 2 to 6.5 and are in the same range than the ones calculated in Caco-2 cells (Alassane-Kpembi *et al.*, 2013).

Biologically, several hypotheses could be proposed to explain the synergistic interaction between TCTB. One hypothesis at membrane level is a differential

interaction of the toxins with efflux pump. TCT B are substrates for P-gp and MRP2 efflux transporters (Tep *et al.*, 2007; Videmann *et al.*, 2007). One could speculate that differential affinity of these TCT B with transporters may contribute to a differential cellular accumulation and the subsequent intrinsic toxicity of the TCT B. 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. At the intracellular target level the synergy could also be due to the activation by the mycotoxin mixtures of new pathways that are not triggered by mycotoxins individually. For example, a genome-wide gene expression approach has been used to elucidate the synergistic cytotoxicity of anti-microtubule agents docetaxel and estramustine on prostate cancer cells, by detecting new targeted genes only affected by both drugs which were not altered by each drug alone (Li *et al.*, 2005). These different hypotheses should be investigated to explain the observed interactions between TCT B.

In conclusion, 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 after the exposure of a non-transformed cell line to the mixtures are biologically relevant since type B trichothecenes often occur in combination. However, besides the *in vitro* results obtained in this study further research concerning the *in vivo* toxicity of mycotoxins mixtures and their interactions are needed to provide regulatory agencies with substantial data in health risk assessment for the actual co-exposure to mycotoxins.

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Competing financial interests declaration: Authors declare no conflict of interest

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Table 1: Dose-effect relationship parameters for viability inhibition by DON, NIV, their acetyl derivatives and binary mixtures in Ipec-1 cells.

Mycotoxin	Dose-effect parameters				
	D_m (μM)	m	r	IC_{10} (μM)	IC_{90} (μM)
DON	3.56	0.90	0.974	0.31	40.62
3-ADON	19.84	0.75	0.973	1.06	370.19
15-ADON	2.41	0.94	0.967	0.24	24.72
NIV	2.11	0.87	0.975	0.17	26.19
FX	2.29	0.96	0.968	0.23	22.34
DON - 3-ADON	12.59	1.00	0.987	1.39	114.34
DON - 15-ADON	1.78	0.84	0.996	0.13	24.14
3-ADON - 15-ADON	7.27	0.76	0.998	0.40	132.60
DON - NIV	2.19	0.88	0.982	0.18	26.87
DON - FX	4.52	0.86	0.984	0.35	58.67
NIV - FX	1.98	0.90	0.986	0.17	22.66

The parameters m , D_m and r are the slope, the median-effect dose, and the linear correlation coefficient which signify the shape of the dose-effect curve, the potency (IC_{50}), and conformity of the data to the mass-action law, respectively (Chou, 2006). IC_{10} and IC_{90} , are the doses required to inhibit cell viability by 10 and 90%, respectively. Computer software CompuSyn was used for automated calculation and simulation.

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

CI are the Combination Index values for the mixtures $CI < 0.9$, $0.9 < CI < 1.1$, and $CI > 1.1$ indicate synergism, additive effect, and antagonism, respectively. Dose reduction indices were calculated by comparing the concentration required to reach 10, 50 and 80% cytotoxicity when the mycotoxin was used singly and in combination. $DRI > 2$ indicate a synergistic effect. Computer software CompuSyn was used for automated calculation.

Figure legends

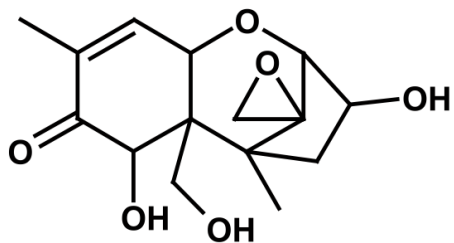
Figure 1: Chemical structures of DON and NIV and their acetyl derivatives

Figure 2: Comparative toxicity of individual mycotoxins (panel A) and DON (○), 3-ADON (◻), 15-ADON (△), NIV (▽) and FX (◐) in binary combinations (◆) on proliferating Ipec-1 cells. Intestinal epithelial cells were exposed for 24 hours to serial dilutions of toxins alone or in combination and cytotoxicity was assessed by the MTT assay. Data are means ± SD of three independent experiments.

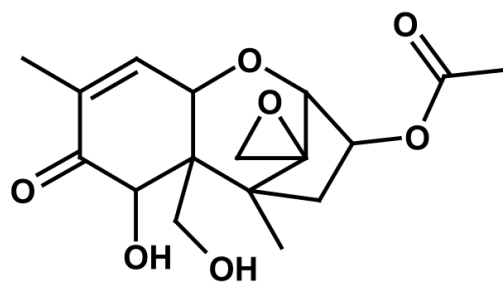
Figure 3: Isobologram illustrating the combined cytotoxicity of DON and 3-ADON. At concentrations eliciting 10% (○), 30% (◼) or 50% (▲) toxicity. The points are mean concentrations of dose-response MTT test cytotoxicity curves for each toxin or toxin combination (Compusyn software analysis).

Figure 4: 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.

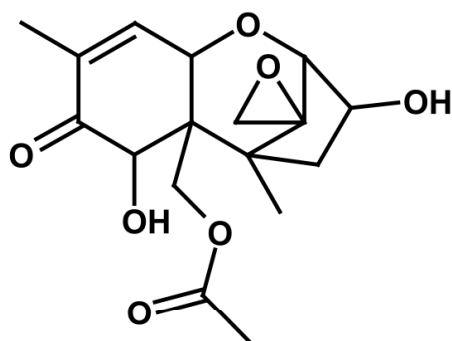
Alassane-Kpembé et al. Figure 1



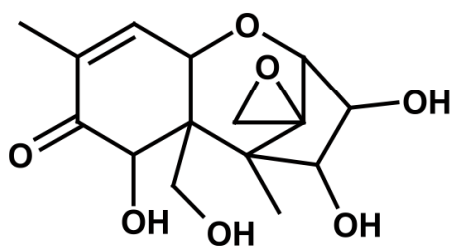
Deoxynivalenol (DON)



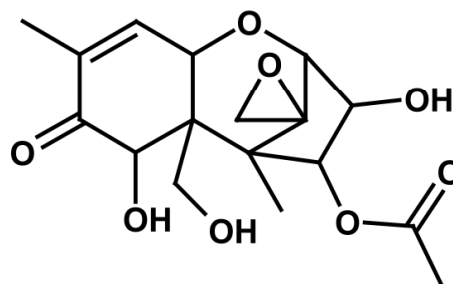
3-acetyldeoxynivalenol (3-ADON)



15-acetyldeoxynivalenol (15-ADON)



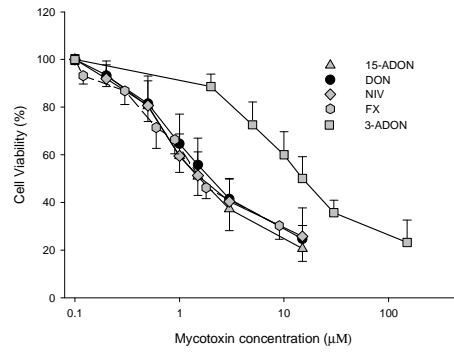
Nivalenol (NIV)



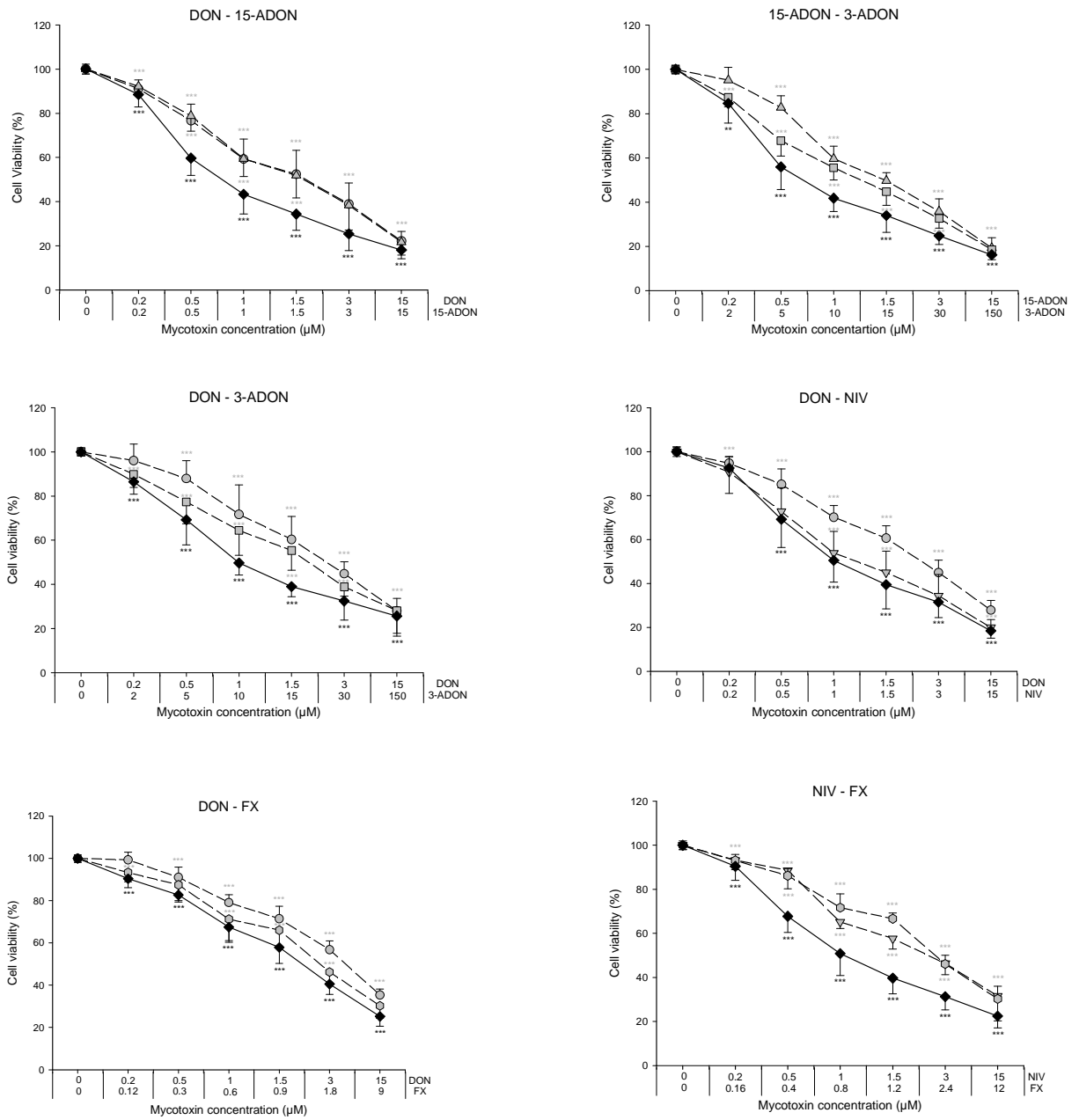
Fusarenon-X (FX)

Alassane-Kpembi et al. Figure 2

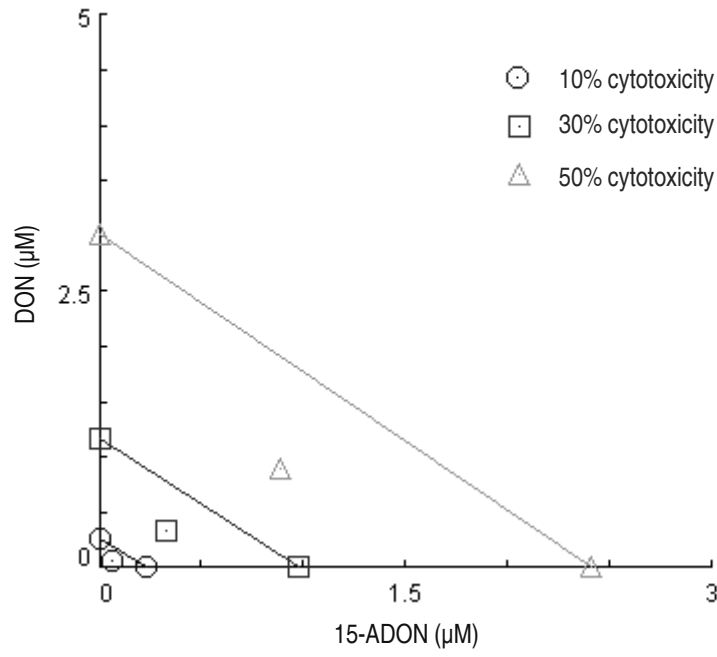
A.



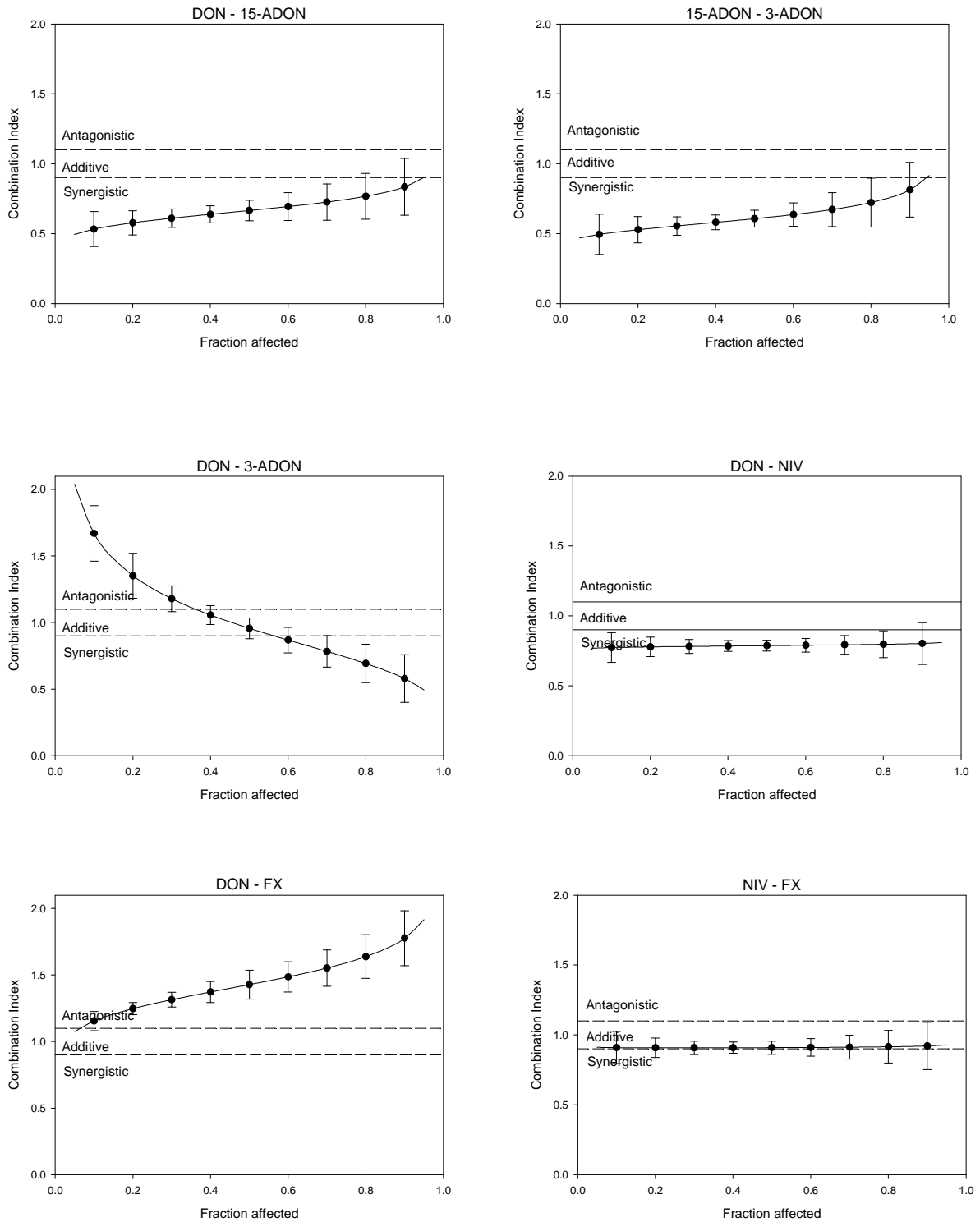
B.



Alassane-Kpembi et al. Figure 3



Alassane-Kpembi et al. Figure 4



CHAPITRE II :

CARACTERISATION DE L'ACTIVITE INTESTINALE PRO-INFLAMMATOIRE
DES TRICHOTHECENES B.

Les résultats de ce travail sont présentés sous forme d'article :

Alassane-Kpembé I., Puel O., and Oswald I.P., 2013. Transcriptional Regulation of the Inflammatory Response in the Intestine upon Exposure to Trichothecene Mycotoxins Deoxynivalenol, Nivalenol and Fusarenon-X: a Benchmark Dose Analysis. *Manuscrit en préparation.*

RESUME DE L'ETUDE

In vitro et *in vivo*, l'activation DON-dépendante de certaines voies de la signalisation cellulaire, dont MAPKinase et NF- κ B, est associée à la mise en place d'une réponse pro-inflammatoire. Un nombre croissant de publications suggèrent même que le DON, à défaut de jouer un rôle central dans l'étiologie probablement multifactorielle des maladies inflammatoires chroniques de l'intestin, pourrait en constituer un facteur aggravant. L'objectif de cette partie de la thèse est de caractériser l'activité pro-inflammatoire intestinale comme marqueur de la toxicité intestinale des TCTs B.

Nous avons analysé dans un système *ex-vivo* d'explants intestinaux de porc les relations dose-effet pour l'activité pro-inflammatoire intestinale du DON, mais également du NIV et de la FX. Les données d'expression génique du facteur de transcription NF- κ B, de la chimiokine IL-8, et des cytokines IL-1 α , IL-1 β , IL-17A, IL-22 et IL-23A ont mis en évidence une activation, dans certains cas bi-phasique, des mécanismes de l'inflammation par les TCTs B étudiés. L'analyse benchmark dose des données de transcriptomique a montré que l'exposition de l'intestin à des doses aussi faibles que 0,04 μ M de FX, 0,1 μ M de DON ou 0,1 μ M de NIV s'accompagne d'une activation significative des mécanismes de l'inflammation. Ces doses sont de l'ordre des concentrations attendues dans le chyle sur la base des valeurs toxicologiques de référence actuelles. Elles montrent donc que l'activité pro-inflammatoire au niveau intestinal pourrait être un marqueur très sensible de la toxicité des TCTs B.

Title: Transcriptional Regulation of the Inflammatory Response in the Intestine upon Exposure to Trichothecene Mycotoxins Deoxynivalenol, Nivalenol and Fusarenon-X: a Benchmark Dose Analysis.

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Abstract

Deoxynivalenol (DON) belongs to type B trichothecenes, a class of secondary metabolites produced by the *Fusarium graminearum* species complex. This group of mycotoxins causes an increasing public health concern, owing to their deleterious effects for human and animal health and their frequent occurrence. At the cellular level, their binding to the ribosome is followed by the activation of the mitogen-activated protein kinase (MAPK) pathways that are implicated in the regulation of key cellular processes including inflammatory responses. Based on the ability of DON to disturb the state of homeostasis of the inflammatory response in the intestine mimicking what is found in inflammatory bowel diseases (IBD), it is proposed that this mycotoxin may play a role in such diseases. Here we analyzed in an *ex-vivo* pig jejunal explants model the dose-response relationships for the intestinal inflammatory effects of DON and two other congeners, nivalenol (NIV) and fusarenon-X (FX). Benchmark dose analyses were conducted using the up-regulations in gene expression of the transcription factor NF- κ B, the cytokines TNF- α , IL-1 α , IL-1 β , IL-17A, IL-22 and IL-23A and the chemokine IL-8 as endpoints. Monotonic and non-monotonic dose-dependent gene up-regulation patterns were observed in the explants upon exposure to the type B trichothecenes. Doses as low as 0.04 μ M FX, 0.1 μ M DON and 0.1 μ M NIV were shown to significantly increase the mRNA expression levels of the inflammation-related genes in the intestine. Considering that these doses are lower than the tolerable daily intake (TDI)-related intestinal concentration for DON and NIV, it is concluded that the intestinal inflammation may be considered a very sensitive and useful endpoint for the trichothecenes risk assessment in the future.

Introduction

Fungi spoiling all stages of the food chain produce a number of secondary metabolites that may act as important factors in the initial colonization of the substrate or provide them with an advantage regarding competition with other microorganisms. Mycotoxins are some of these metabolites that have been identified for their deleterious effects for human and animal health. Among them, type B trichothecenes produced by the *Fusarium graminearum* species complex are considered the most prevalent in crop production in Europe and North America (CAST, 2003; Schothorst and van Egmond, 2004). Two main chemotypes of type B trichothecene-producing strains are recognized (Ward *et al.*, 2002). Inside the chemotype I two sub-chemotypes Ia and Ib produce deoxynivalenol (DON) and either its acetyl derivatives 3-acetyldeoxynivalenol or 15-acetyldeoxynivalenol, while chemotype II produces nivalenol (NIV) and its acetyl derivative fusarenon-X (FX).

The adverse effects of trichothecenes include emesis, nausea, anorexia, growth retardation, neuroendocrine changes and immunosuppression (Pestka, 2010b). At the molecular level, it has been shown that the capacity of trichothecenes to inhibit protein synthesis upon their binding to ribosomal RNA is pivotal for their *in vivo* and *in vitro* effects (Ueno, 1984; He *et al.*, 2012). Like many other ribotoxins, the binding and cleavage of ribosomal RNA by trichothecenes, especially DON, rapidly activate mitogen-activated kinases (MAPKs) via a so-called “ribotoxic stress response” (Iordanov *et al.*, 1997; Shifrin and Anderson, 1999; Zhou *et al.*, 2003). MAPKs have been implicated in the regulation of key cellular processes including gene induction, cell survival/apoptosis, proliferation and differentiation as well as cellular stress and inflammatory responses (Thalhamer *et al.*, 2008). Inflammation is a complex set of interactions among soluble factors and cells that can arise in any tissue in response to infectious or non-infectious injury. This process normally leads to recovery from infection and to healing. However, if targeted destruction and assisted repair are not properly phased, inflammation can lead to persistent tissue damage. For a number of

chronic diseases characterized by a persistent inflammation of the gut, the role of intestinal inflammation as a cause or a consequence of tissue destruction is still being debated. The primary MAPK subfamilies are the extracellular signal-regulated protein kinases (ERK1 and 2), the c-Jun N-terminal kinases (JNK 1 and 2), and p38 MAPK (Widmann et al., 1999). ERK and p38 have been associated to the DON-induced trans-activation of a number of pro-inflammatory cytokines and the increase of their mRNA stability as well (Pestka, 2008). Exposure to DON at 10 μ M also caused a profound alteration of the intestinal pathogenic Th-17 inflammatory response at the expenses of regulatory/protective Th-17 response (Cano et al., 2013). Based on the ability of DON to cause intestinal immune alterations mimicking the one found in inflammatory bowel diseases (IBD), it is proposed that DON may play a role in such diseases (Maresca and Fantini, 2010; Cano et al., 2013; Maresca, 2013).

Using a transcriptional benchmark dose analysis approach, the aim of this study was to characterize the dose-response relationship of the intestinal pro-inflammatory activity of DON, but also NIV and its acetyl derivative FX.

2. Material and methods

2.1 Chemicals

Purified deoxynivalenol (DON) purchased from Sigma (St Quentin Fallavier, France) and nivalenol (NIV) and fusarenon-X (FX) purchased from Waco Pure Chemical Industries LTD (Osaka, Japan) were dissolved in dimethylsulfoxide (DMSO) to 60 mM, and stored at -20°C before dilution in complete culture media. Control samples were treated with equivalent concentrations of DMSO, which were non toxic.

2.2 Treatment of explant cultures

All animal experiments were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. Jejunal tissue was obtained from

six piglets which were 5 week-old, 7–days after weaning. Animals were fed *ad libitum* prior to the experiment. 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 gentamycin (Eurobio), 4.5 g/L of glucose (Sigma), 10% FBS (Eurobio) and 30 mM of amino acid (Ala/Glu) (Eurobio). Explants were exposed to purified DON or NIV at 0, 0.1, 0.3, 1, 3, 10 and 30 µM, or purified FX at 0, 0.03, 0.1, 1, 3, 10 and 30 µM, and incubated at 39°C in a humidified atmosphere of 5% CO₂ for 5 hours. After treatment, tissues were collected for transcriptional analysis.

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

Quantitative real-time PCR (qPCR) was performed to determine the relative mRNA expression levels of chemokines, pro-inflammatory cytokines, the NF-κB transcription factor, and the porcine β-defensins 1 and 3 involved in the modulation of the intestinal immune response and the regulation of the local antimicrobial defense. RNA concentration, integrity and quality were determined spectrophotometrically using Nanodrop ND1000 (Labtech International, Paris, France) and analysis of the electrophoregram generated by an Agilent Bioanalyzer. RNA Integrity Numbers (RIN) of these mRNA were around 6.10. Then reverse transcription and real-time qPCR steps were performed as previously described (Meissonnier et al., 2008). Non-reverse transcribed 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 synthesized and purified by 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. Both genes had a stable expression, in all the samples tested, as calculated using the Bestkeeper-1 software (Pfaffl *et al.*, 2004) (data not shown). 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. This approach overcomes the problems that make very difficult to perform traditional statistical analysis, such as the absence of standard deviation in ratio distributions (Pfaffl *et al.*, 2002).

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

A Benchmark dose analysis of the point of departure (POD) for the different endpoints was conducted to compare the pro-inflammatory potency of DON, NIV and FX. The Benchmark dose (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. One standard deviation above the control mean was used to set the BMR as suggested by U.S. EPA (1995). Assuming pro-inflammatory cytokine gene expressions above the 99th percentile of the control mean (control mean + 2.33 standard deviations) are considered adverse, this benchmark response identifies a mycotoxin dose at which 10% of treated explants would have pro-inflammatory cytokines gene expression above the 99th percentile (Crump, 1995).

For determining BMDs of all the tested mycotoxins the software PROAST (Possible Risk Obtained from Animal Studies) was used. This procedure is extensively described elsewhere (Muri *et al.*, 2009). The program has been developed by the Dutch National Institute for Public Health and the Environment (RIVM) in Bilthoven and is available from the RIVM website (<http://www.rivm.nl/proast>). In brief PROAST consists of five nested models with increasing complexity. It calculates for every model a numerical value called log-likelihood that expresses relatively the

goodness of fit of the model to the data. When a model is extended by one or more parameters, the resulting log-likelihood achieves a higher or equal value compared to the model with fewer parameters. A model with a given number of parameters is chosen if its extension by increasing the number of parameters does not result in a statistically significant higher log-likelihood. That procedure based on the statistical likelihood ratio test allows a formal selection of the best fitting model which was used to calculate the BMD and the benchmark dose lower confidence bound (BMDL).

3. Results

3.1 DON, NIV and FX dose-dependently up-regulate the expression of pro-inflammatory cytokines

We assessed the pro-inflammatory activity of the type B trichothecenes DON, NIV or FX by measuring the gene expression for the NF- κ B transcription factor and cytokines IL-1 α , IL-1 β , IL-8 and TNF- α in pig jejunum explant cultures incubated with graded levels of the mycotoxins.

After 5 hours, all these mycotoxins induced a dose-dependent up-regulation in the mRNA expression of the transcription factor NF- κ B (Fig 2 panel A). The explant cultures exposed to the highest DON and FX concentrations (30 μ M) showed a 4-fold mean increase for the NF- κ B gene expression ratio. No observed adverse-effects levels (NOAELs) were 0.3 μ M for FX and 1 μ M for DON and NIV.

Globally, the pro-inflammatory cytokines IL-1 α , TNF- α , IL-1 β and IL-8 showed higher levels for gene activation than the transcription factor NF- κ B (Fig. 1 panels A & B). Two different regulation patterns could be noted. IL-1 α and TNF- α demonstrated a constant dose-dependent up-regulation, while a bell-shaped activation was observed for IL-1 β and IL-8. Indeed for the latter cytokines, the upward trend of the genes activation for graded levels of the mycotoxins up to 3-10 μ M was followed by a decrease. The lowest NOAELs were observed with FX for IL-8 (0.1 μ M), and for

IL-1 α and TNF- α (0.3 μ M). Concerning IL-1 β , the same value for NOAEL (0.1 μ M) was reported for all DON, NIV and FX.

3.2 DON, NIV and FX dose-dependently up-regulate the pathogenic Th-17 response

In order to evaluate the adaptative inflammatory response, and especially the pathogenic Th-17 response of the intestine following exposure to the three type B trichothecenes, we analyzed the mRNA expression levels for cytokines IL-17A, IL-22 and IL-23A in pig jejunum explants cultures exposed to graded concentrations of the mycotoxins. The results are presented in Fig 3.

After the 5-hour incubation, all the three mycotoxins dose-dependently up-regulated the gene expression of IL-17A, with a clear bell-shaped activation for DON and FX. At the highest activation level, an eleven-fold change in IL-17A gene expression was observed with DON. DON and FX presented a lower NOAEL (0.3 μ M) than NIV (1 μ M). A bell-shaped activation of IL-22 gene by DON was also reported, with a maximum of 7-fold change in gene expression observed when explants were exposed to 10 μ M. The NOAELs for IL-22 gene up-regulation were 0.3 μ M for DON and FX, and 1 μ M for NIV.

By contrast to IL-17A and IL-22 gene expressions, a very slight and not significant up-regulation in the IL-23A gene expression could be reported for increasing concentrations of DON and NIV. Statistical significance in gene expression up-regulation was reached only for 10 μ M and 30 μ M concentrations of FX.

3.3 Benchmark dose analysis of the pro-inflammatory potency of DON, NIV and FX.

In order to rank the pro-inflammatory potencies of DON, NIV and FX, we compared their threshold points for the up-regulation of pro-inflammatory cytokines and cytokines involved in the pathogenic Th-17 response. Benchmark dose analyses were performed to derive the PODs associated to cytokine gene up-regulation for each mycotoxin. Two different families of dose-response models (exponential and Hill) were fit to the mRNA expression data. For each family of dose-response models, a nested likelihood ratio test was performed to select the best fitting model using PROAST software. This model was used to calculate the BMD and its lower confidence bound (BMDL). Examples of the selected fitting models for the cytokine mRNA expression data are presented in supplemental materials.

The BMD and BMDL values derived for the up-regulation of the pro-inflammatory cytokine IL-1 β and the chemokine IL-8 by DON, NIV and FX are presented in Table 2. No difference could be noted between the potencies of DON and NIV for the up-regulation of the cytokine IL-1 β , and concentrations as low as 0.25 μ M constituted the threshold for the gene activation. DON showed a higher potency of up-regulation for chemokine IL-8 compared to NIV. For both cytokine up-regulations, FX demonstrated higher potency than DON and NIV, and its threshold concentration was globally lowered by a mean factor 2 compared to DON.

Table 3 presents the BMDs and BMDLs associated to the pathogenic Th-17 response. Globally the same ranking in potency for DON, NIV and FX was observed for the adaptative inflammatory response, but the up-regulation of the pathogenic Th-17 cytokines appeared less sensitive to the effects of the trichothecenes tested, especially NIV. The lowest BMDL observed for the IL-17A – IL-22 up-regulation by NIV was 0.7 μ M, compared to the 0.1 μ M observed for the pro-inflammatory cytokines.

4. Discussion

Trichothecenes, especially DON, raise increasing concern for food safety issues, given the growing body of evidences of their potential to cause toxicity in humans and animals and their frequent and global worldwide occurrence. Surveys estimated that 98% of the adults in the United Kingdom had been exposed to DON, and 80% of children in the Netherlands exceeded the tolerable daily intake (TDI) for this mycotoxin (Pieters *et al.*, 2002; Turner *et al.*, 2008). The intestine that is the first barrier against food contaminants shows high sensitivity to the toxicity of DON and related mycotoxins (Pinton *et al.*, 2010; Bracarense *et al.*, 2012; Pinton *et al.*, 2012; Lucioli *et al.*, 2013). Our lab recently reported the ability for DON at 10 μ M to disrupt the intestinal homeostasis of the inflammatory response and to promote the Th-17 response over the Th-1 and T-regulatory ones (Cano *et al.*, 2013). Here we explored these effects at dietary concentrations for other type B trichothecenes additionally to DON.

In this study, the tested concentrations for trichothecenes effects ranged from 0.03 μ M to 30 μ M. The European Food Safety Authority established a tolerable daily intake (TDI) of 1.2 μ g/Kg of body weight (BW) per day for NIV (EFSA CONTAM, 2013). The provisional mean tolerable daily intake (PTMDI) of DON has been set to 1 μ g/Kg of BW per day (JECFA, 2011). Assuming that a human adult weighs 70 Kg and has a global small intestinal volume of 1L, it is considered that this PTMDI would correspond to a toxin concentration in the intestinal lumen of 0.21 μ M (Maresca, 2013). Likewise, the TDI for NIV would correspond to an intestinal concentration of 0.25 μ M. On the basis of the worldwide higher exposure in adult and children to DON obtained from the FAO/WHO Expert Committee on Food Additives (JECFA), this concentration would reach maximal values of 0.5 μ M. However concentrations up to 30 μ M should also be considered as still realistic but encountered more occasionally, in case for instance of severe *Fusarium* infection of cereals following unfavorable weather conditions (Larsen *et al.*, 2004; Van De Walle *et al.*, 2008; Maresca, 2013).

In this study, the regulations of the transcription factor NF- κ B, the cytokines TNF- α , IL-1 α and IL-1 β and the chemokine IL-8 were used as endpoints for the analysis of the effects of trichothecenes on the innate inflammatory response. The ability of trichothecenes to induce immune- and inflammation-associated genes is well established and at least two mechanisms have been proposed to explain how DON could up-regulate gene expression (Pestka, 2010a). DON increases binding of several transcription factors, especially NF- κ B involved in inflammation pathways. DON also stabilizes the mRNA transcripts of number of inflammation genes including TNF- α , IL-2, IL-6, IL-8 and COX-2. The nuclear factor- κ B (NF- κ B) signaling pathway regulates immune responses and is implicated in the pathogenesis of many inflammatory diseases (for comprehensive reviews Pasparakis, 2009; Wullaert *et al.*, 2011). The NF- κ B family of transcription factors consists of five members in mammals, namely p65/RelA, RelB, c-Rel and the p50 and p52 proteins. NF- κ B proteins form homo- or heterodimers that can bind to consensus DNA sequences on the regulatory regions of target genes and regulate their transcription. In resting cells, NF- κ B dimers are kept inactive by association with inhibitory proteins of the I κ B family. Upon stimulation, the I κ B kinase (IKK) phosphorylates I κ B proteins on specific serine residues, which induces their subsequent polyubiquitylation and proteasomal degradation, allowing NF- κ B dimers to accumulate in the nucleus and activate gene transcription. Although the role of DON for increase in nuclear binding of the transcription factor NF- κ B and the subsequent pro-inflammatory genes induction has been confirmed by several studies, the mechanism by which such binding increases remains unclear (Wong *et al.*, 2002; Maresca *et al.*, 2008; Van De Walle *et al.*, 2008). DON-mediated inhibition of re-synthesis of the cytoplasmic inhibitor I κ B α is proposed to explain in part the increased binding activity of NF- κ B/Rel (Pestka *et al.*, 2004). Based upon the observation that following DON exposure, p65 binding significantly increases while p52 binding decreases and p50 remains constant, a DON-induced shift in the composition of NF- κ B was also suggested (Gray and Pestka, 2007). Exposing the intestinal mucosa to realistic

concentrations of DON, but also NIV and FX, we observed a dose-related induction of the NF- κ B 1 gene encoding the p50 protein. For the best of our knowledge, this is the first report of a dose-dependent expression of NF- κ B mRNA transcripts following exposure to trichothecenes. Taken together these observations evoke at least a transcriptional component in the regulation of the NF- κ B signaling pathway by trichothecenes, rather than the simple protein synthesis inhibition of a cytoplasmic inhibitor. It should be further noted that DON implication in the regulation of the gene expression of other transcription factors (e.g., c-Fos, Fra-2, c-Jun, Jun B, EGR1, ATF3) has already been demonstrated (Kinser *et al.*, 2005; Nielsen *et al.*, 2009).

Two regulation patterns by trichothecenes DON, NIV and FX were observed for pro-inflammatory cytokines. IL-1 α and TNF- α demonstrated a constant dose-dependent up-regulation, while a bell-shaped activation was observed for IL-1 β and IL-8. A biphasic effect was previously reported in human intestinal epithelial cells, with DON doses up to 25 μ M causing a massive increase of IL-8 secretion whereas higher doses presented inhibitory effects (Maresca *et al.*, 2008). We also observed that the pathogenic Th-17 genes IL-17A and IL-22 were non-monotonically induced by trichothecenes. This may be linked to the two feed-back mechanisms downscaling pro-inflammatory gene effects that have been described for DON in a mouse models: (i) the induction of several suppressors of cytokine signaling (SOCS) notably SOCS1, SOCS2, SOCS3 and CIS (cytokine-inducible SH2 domain protein) (Amuzie *et al.*, 2009), and (ii) the up-regulation of mitogen-activated protein kinase phosphatase 1 (MKP1) (Kinser *et al.*, 2004).

Contrary to IL-17A and IL-22 gene expressions, a very slight and not significant up-regulation in the IL-23A gene expression could be reported in this study for increasing concentrations of DON and NIV. This may be explained by the delayed time-course of IL-23A induction compared to IL-17A and IL-22, as shown in an aforementioned work (Cano *et al.*, 2013).

We used benchmark dose analysis of genomic data to determine transcriptional points of departure for trichothecenes' risk assessment. Few examples of such practical application exist even though a number of studies are questioning the transcriptional sounding of the toxicity of trichothecenes (Diesing *et al.*, 2012; Katika *et al.*, 2012; Wang *et al.*, 2012; He *et al.*, 2013; Osselaere *et al.*, 2013). The primary advantages in applying genomic technology to risk assessment are the sensitivity and comprehensive examination of the molecular changes resulting from chemical exposure (Thomas *et al.*, 2011). The NOAEL is traditionally used as a starting point in the determination of guidance values in the health-risk assessment of the non-genotoxic agents. It is defined as the highest experimental dose level for which the response is not significantly different compared with the response in the control group. This approach presents two main limitations (Thomas *et al.*, 2007): (i) the final NOAEL can heavily rely on the dose spacing and the experimental sample size, and (ii) the NOAEL does not account for the variability in the estimate of the dose-response or the slope of the dose-response curve. The dependency on dose spacing also makes it difficult to compare NOAELs across chemicals or studies. Moreover, if a study does not utilize appropriate dose levels or spacing, the ability of the NOAEL approach to determine a POD that approximates a low response is compromised (Davis *et al.*, 2011). The lower confidence limit of the BMD (BMDL) has been proposed to replace the NOAEL as a starting point for determination of health based guidance values (Sand *et al.*, 2008). The application of BMD analysis provide several advantages including better use of dose-response information, more appropriate reflection of experimental size and the lack of constraint to experimental doses (Filipsson *et al.*, 2003). Thus, this approach is gaining more consideration from regulatory agencies addressing such issues (U.S. EPA, 1995; EFSA, 2009).

Globally for the PODs determined here for the effects of trichothecenes DON, NIV and FX towards the intestinal innate and adaptative inflammatory response were clearly below the micromolar range. Until now, the known effective doses for DON on

critical functions of the intestine are estimated to be greater than 1 μM (Maresca, 2013). Our results indicate that intestinal inflammation could be a very sensitive endpoint for the health risk assessment of the trichothecenes. It is also considered that DON may have very low safety margins for intestinal physiological alterations considering its PTMDI/highest exposure-related intestinal concentrations. Here we showed that the current reference doses derived for DON and NIV may not be considered protective. Moreover the risk could be even higher due to the frequent co-occurrence of trichothecenes in food that may result in additive or synergistic toxicological effects (Eckard *et al.*, 2011; Schollenberger *et al.*, 2012; Alassane-Kpembé *et al.*, 2013). Thus food safety regulation agencies should be aware of the need of a reevaluation of the health risk associated to dietary exposure to trichothecenes taking into account more sensitive endpoints and combined exposure patterns.

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TRAVAIL EXPERIMENTAL

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 R: AGCCCCGGTGCCATGT	NC_010445.3 Cano <i>et al.</i> (2013)
IL-1β	Interleukin 1 - beta	F: GAGCTGAAGGCTCTCCACCTC R: ATCGCTGTCATCTCCTTGAC	NM_001005149 Cano <i>et al.</i> (2013)
IL-8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC R: AAGGTGTGGAATGCGTATTTATGC	NM_213867 Bracarense <i>et al.</i> (2012)
IL-17A	Interleukin 17 - alpha	F: CCAGACGGCCCTCAGATTAC R: GGTCCCTCGTTGCGTTGGA	NM_001005729.1
IL-22	Interleukin 22	F: AAGCAGGTCCTGAACTTCAC R: CACCCTTAATACGGCATTGG	AY937228 Levast <i>et al.</i> (2010)
IL-23A	Interleukin 23 - alpha	F: AGAAGAGGGAGATGATGAGACTACA R: GGTGGATCCTTTGCAAGCA	Meurens <i>et al.</i> (2009)
TNF-α	Tumor necrosis factor - alpha	F: ACTGCACTTCGAGGTTATCGG R: GGCGACGGGCTTATCTGA	NM_214022 Meissonnier <i>et al.</i> (2008)
NF-κB	Nuclear factor- κ B 1	F: CCTCCACAAGGCAGCAAATAG R: TCCACACCGCTGTCACAGA	ENSSSCT00000033438
RPL32	Ribosomal Protein L32	F: AGTTCATCCGGCACCAGTCA R: GAACCTTCTCCGCACCCTGT	MN_001001636 Pinton <i>et al.</i> (2010)
β2-Microglob	β 2-Microglobulin	F: TTCTACCTTCTGGTCCACACTGA R: TCATCCAACCCAGATGCA	L13854 Hyland <i>et al.</i> (2006)

Table 2: Benchmark doses for the intestinal up-regulation of the pro-inflammatory cytokine IL-1 β and the chemokine IL-8 by DON, NIV and FX

Cytokines	Mycotoxins	Exponential family models		Hill family models	
		<i>BMD</i> (μ M)	<i>BMDL</i> (μ M)	<i>BMD</i> (μ M)	<i>BMDL</i> (μ M)
IL-1 β	DON	0.24	0.13	0.23	0.12
	NIV	0.23	0.1	0.22	0.09
	FX	0.14	0.04	0.11	0.05
IL-8	DON	0.39	0.22	0.37	0.2
	NIV	1.01	0.54	1.03	0.52
	FX	0.14	0.08	0.24	0.11

Pig jejunal explants were exposed for 5 hours to graded levels of either DON, NIV or FX. Dose-response data of the pro-inflammatory cytokines mRNA expression levels in the explants were fit using exponential and Hill continuous models. The BMDs refer to the mycotoxin concentrations required to cause an increase in mRNA expression one control standard deviation above the control mean. BMDLs refer to lower 95% confidence limit of the corresponding BMDs.

Table 3: Benchmark doses for the intestinal up-regulation of the pathogenic Th-17 response-related cytokines IL-17a and IL-22 by DON, NIV and FX

Cytokines	Mycotoxins	Exponential family models		Hill family models	
		<i>BMD</i> (μM)	<i>BMDL</i> (μM)	<i>BMD</i> (μM)	<i>BMDL</i> (μM)
IL-17a	DON	0.32	0.15	0.32	0.14
	NIV	3.26	2.56	2.49	1.46
	FX	0.16	0.06	0.15	0.05
IL-22	DON	0.89	0.6	0.8	0.52
	NIV	1.65	0.84	1.45	0.69
	FX	0.54	0.34	0.5	0.33

Pig jejunal explants were exposed for 5 hours to graded levels of either DON, NIV or FX. Dose-response data of the pathogenic Th-17 response-related cytokines mRNA expression levels in the explants were fit using exponential and Hill continuous models. The BMDs refer to the mycotoxin concentrations required to cause an increase in mRNA expression one control standard deviation above the control mean. BMDLs refer to lower 95% confidence limit of the corresponding BMDs.

Figure legends

Figure 1: Chemical structures of DON, NIV and FX

Figure 2: DON, NIV and FX dose-responses for the regulation of the NF- κ B 1 gene (panel A) and the pro-inflammatory genes (panel B) expressions in the pig intestine. Jejunal explants from 6 piglets were exposed for 5 hours to graded levels of trichothecenes mycotoxins. The amounts of the target mRNAs were measured by quantitative RT-PCR and analyzed using REST© 2009 software (Qiagen, Valencia, CA). *β 2-Microglobulin* and *RPL-32* were used as the internal references. Data are means \pm SEM (n=6 animals). Symbols *, **, *** 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: DON, NIV and FX dose-responses for the regulation of the pathogenic Th-17 response-related genes expression in the pig intestine. Jejunal explants from 6 piglets were exposed for 5 hours to graded levels of trichothecenes mycotoxins. The amounts of the target mRNAs were measured by quantitative RT-PCR and analyzed using REST© 2009 software (Qiagen, Valencia, CA). *β 2-Microglobulin* and *RPL-32* were used as the internal references. Data are means \pm SEM (n=6 animals). Symbols *, **, *** 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).

Supplemental Material

Figure S1: BMD modeling of the intestinal NF- κ B gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

Figure S2: BMD modeling of the intestinal TNF- α gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

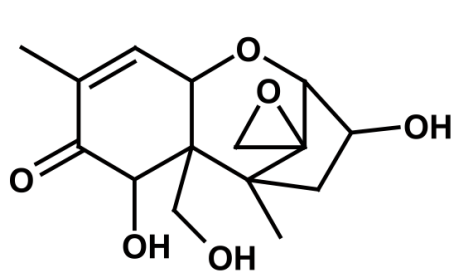
Figure S3: BMD modeling of the intestinal IL-1 β gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

Figure S4: BMD modeling of the intestinal IL-8 gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

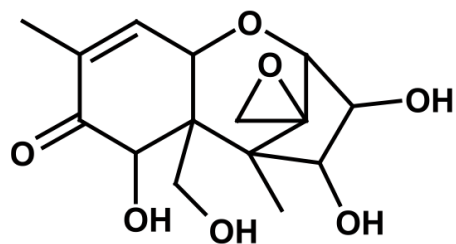
Figure S5: BMD modeling of the intestinal IL-17A gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

Figure S6: BMD modeling of the intestinal IL-22 gene expression data upon exposure to the trichothecenes DON, NIV, and FX using exponential family models (panel A) and Hill family models (panel B).

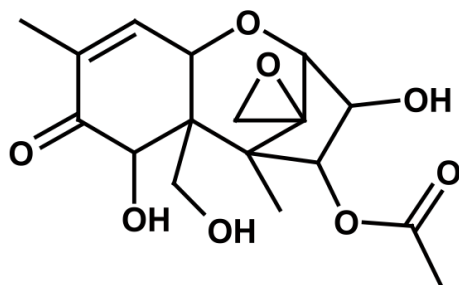
Alassane-Kpembi et al. Figure 1



Deoxynivalenol (DON)



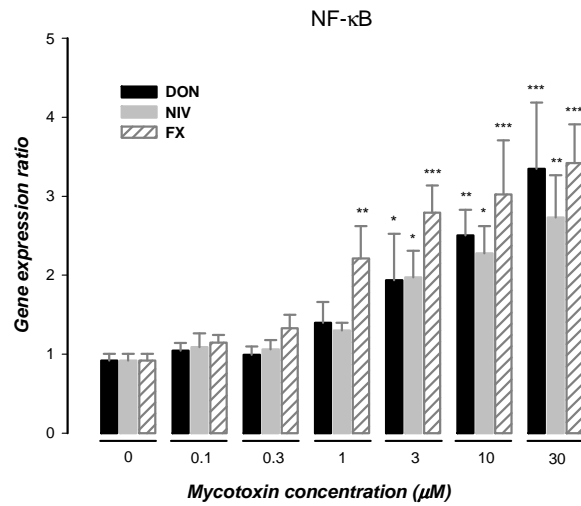
Nivalenol (NIV)



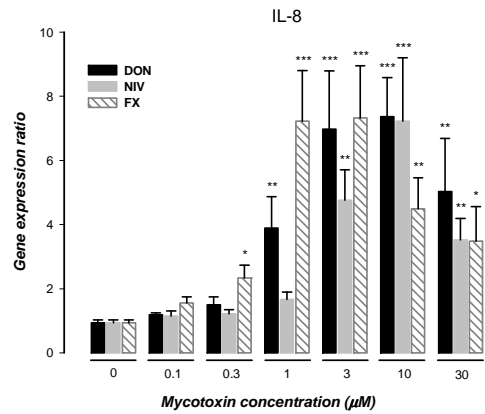
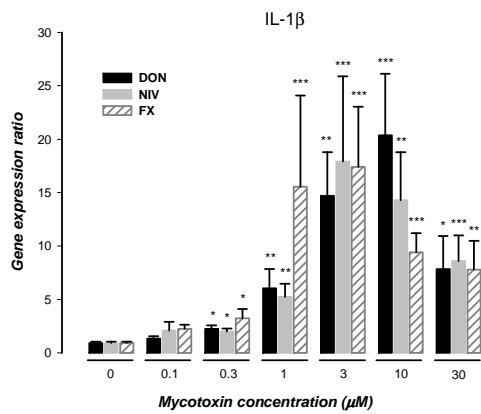
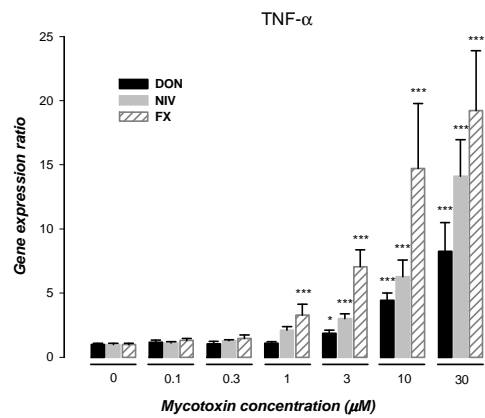
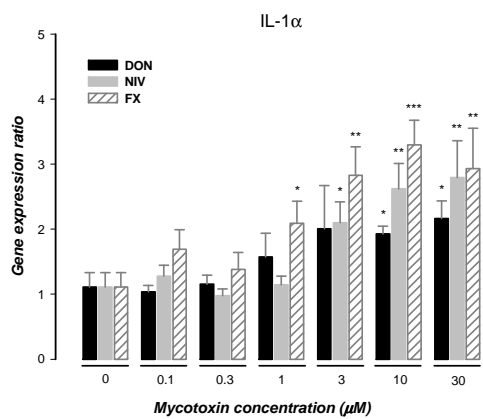
Fusarenon-X (FX)

Alassane-Kpembé et al. Figure 2

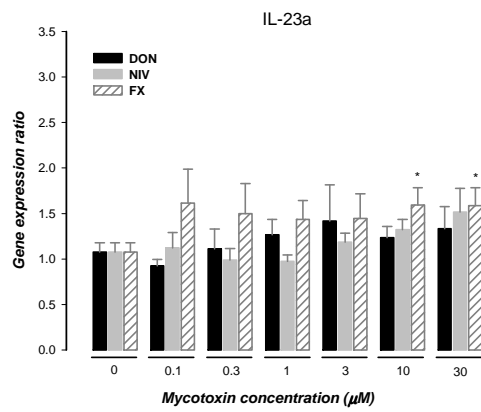
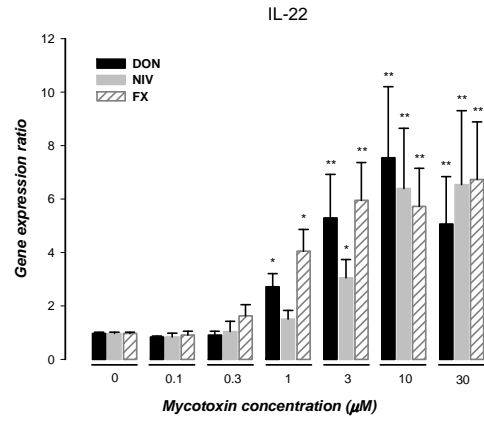
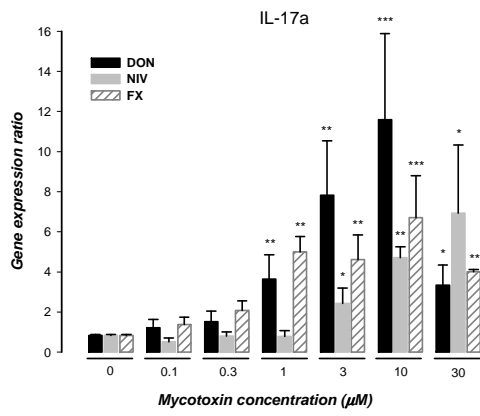
A.



B.



Alassane-Kpembé et al. Figure 3



Supplemental Material

Transcriptional Regulation of the Inflammatory Response in the Intestine upon Exposure to Trichothecene Mycotoxins Deoxynivalenol, Nivalenol and Fusarenon-X: a Benchmark Dose Analysis.

Imourana Alassane-Kpembia^{a,b,c,d}, François A. Abiola^c, Olivier Puel^{a,b}, Isabelle P. Oswald^{a,b}

Supplemental Material, Figure S1 page 2

Supplemental Material, Figure S2 page 3

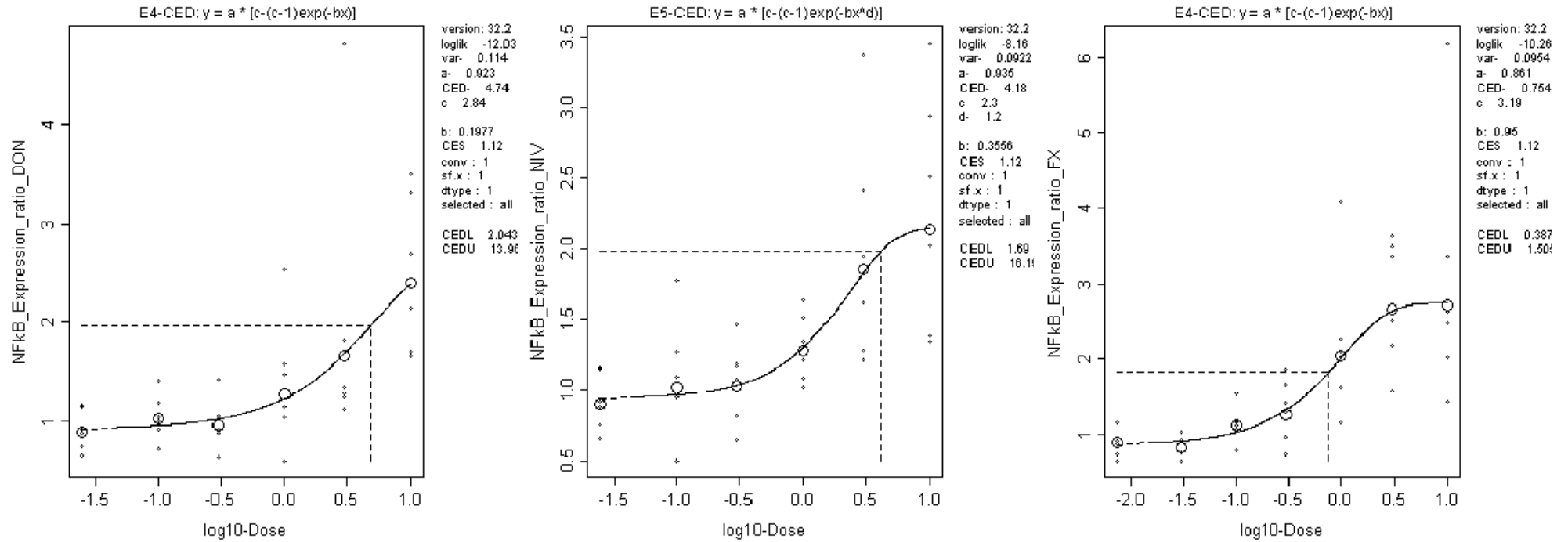
Supplemental Material, Figure S3 page 4

Supplemental Material, Figure S4 page 5

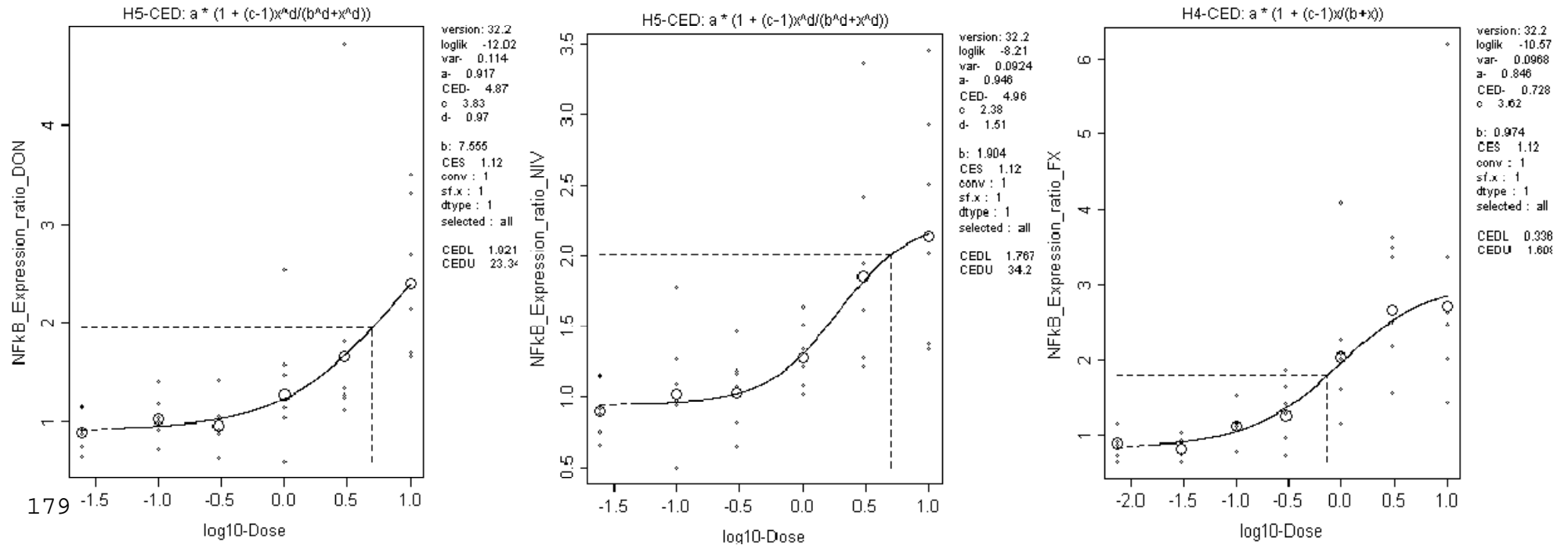
Supplemental Material, Figure S5 page 6

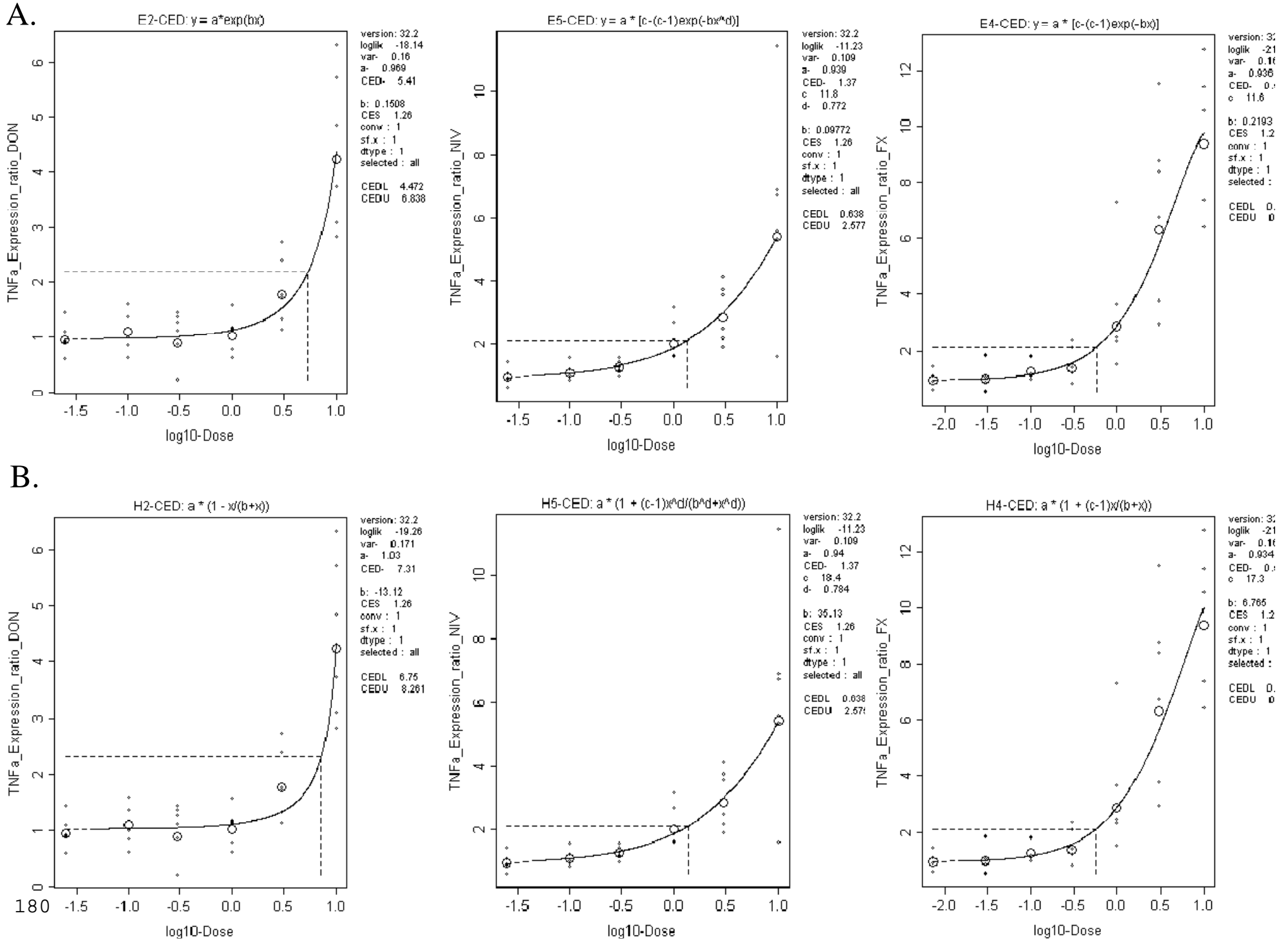
Supplemental Material, Figure S6 page 7

A.



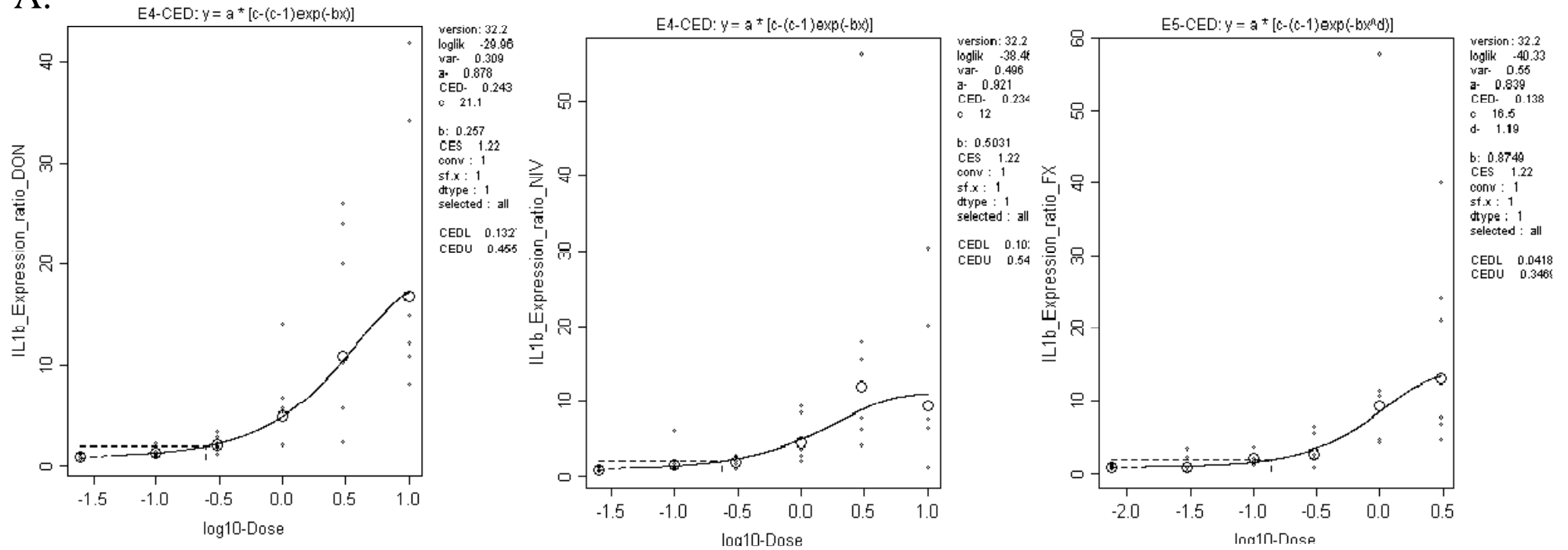
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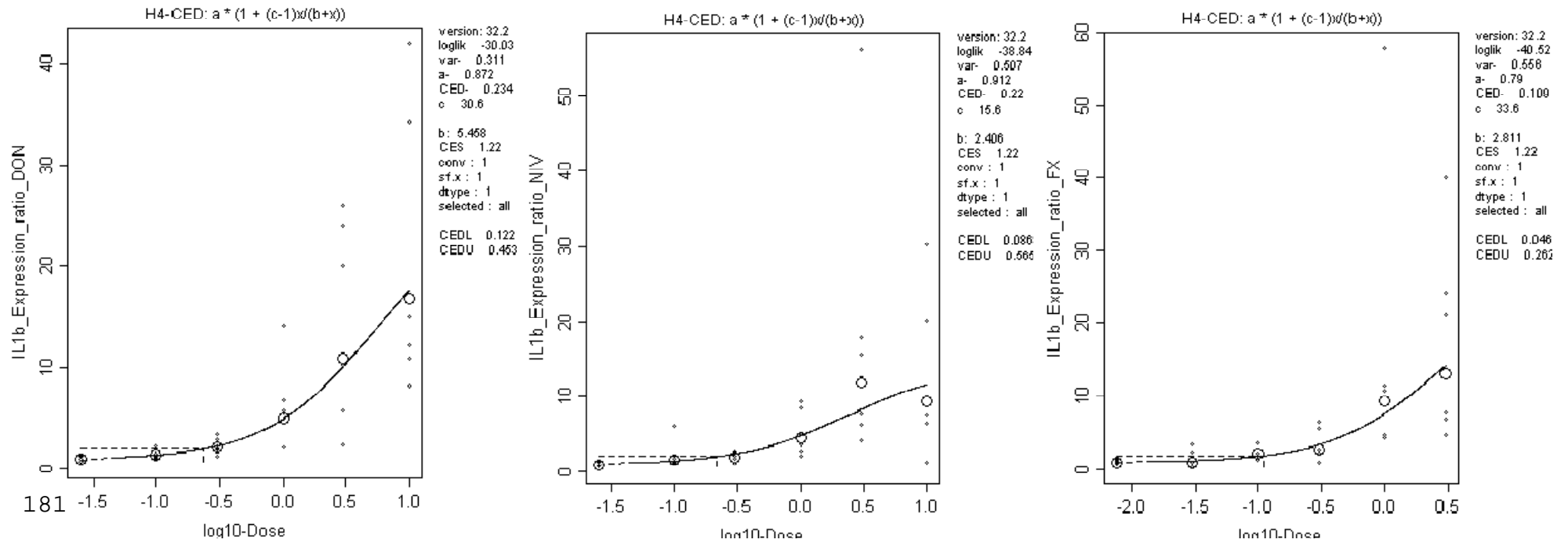


Alassane-Kpembi et al. Fig. S3

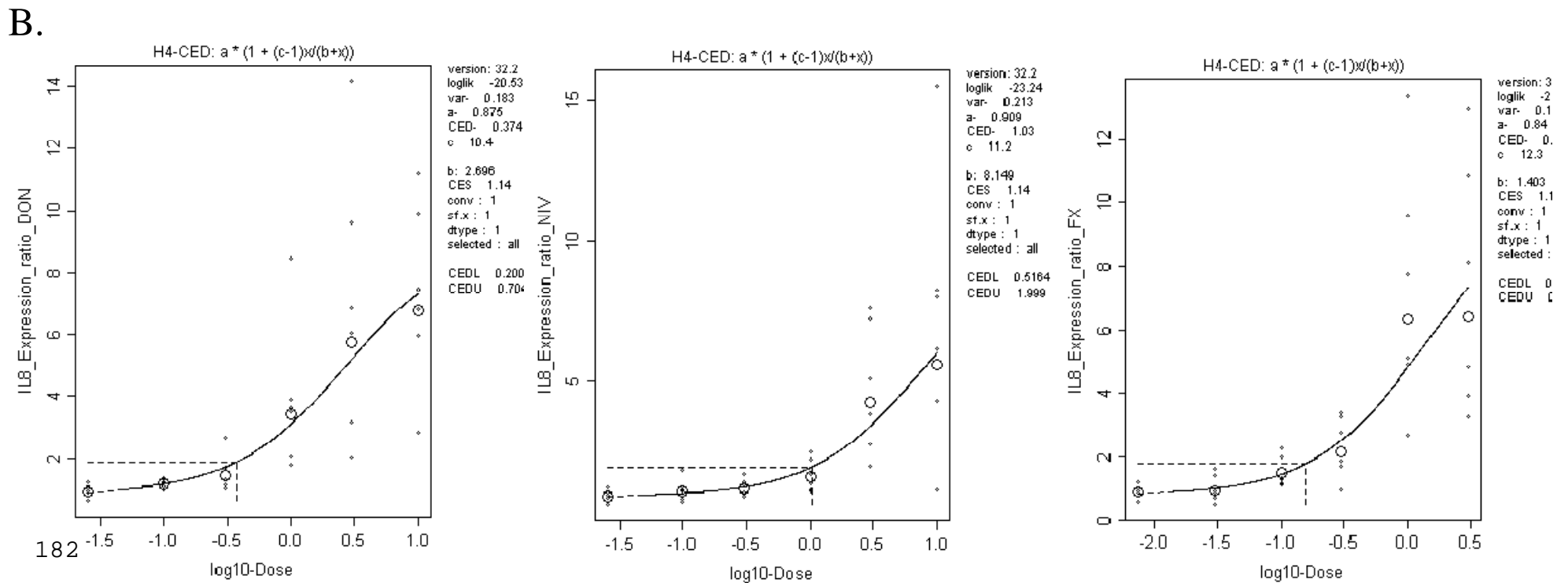
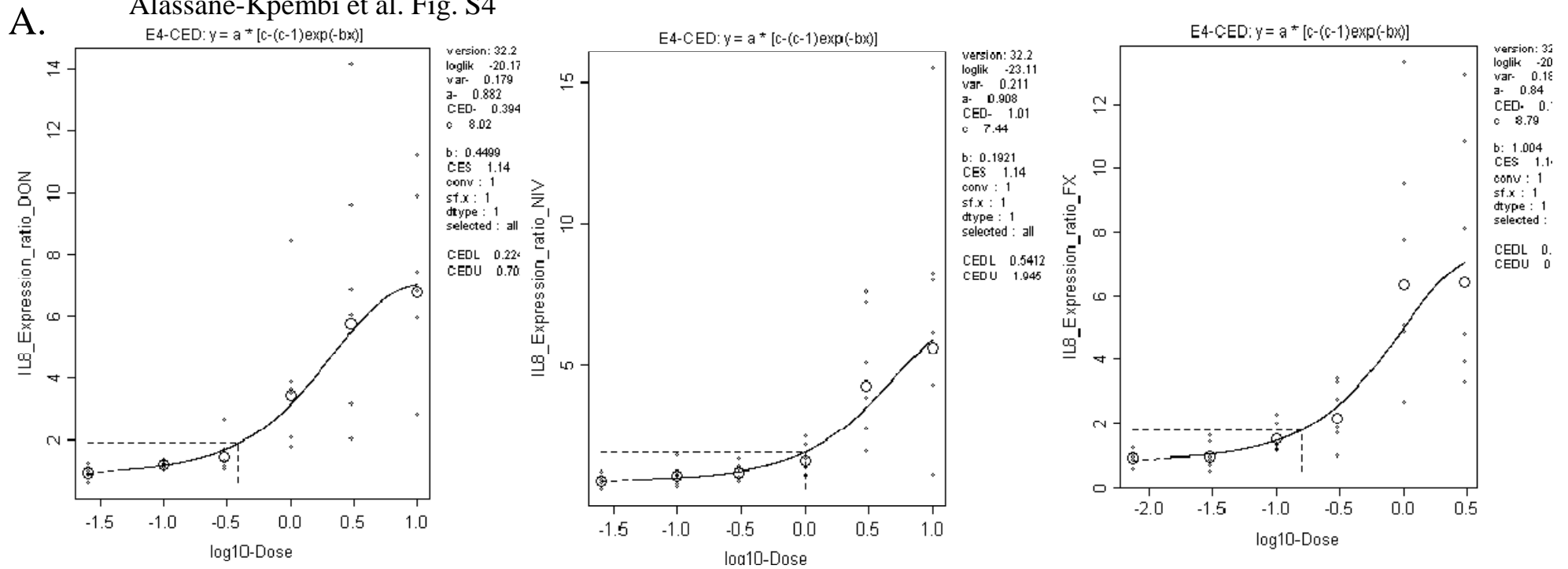
A.

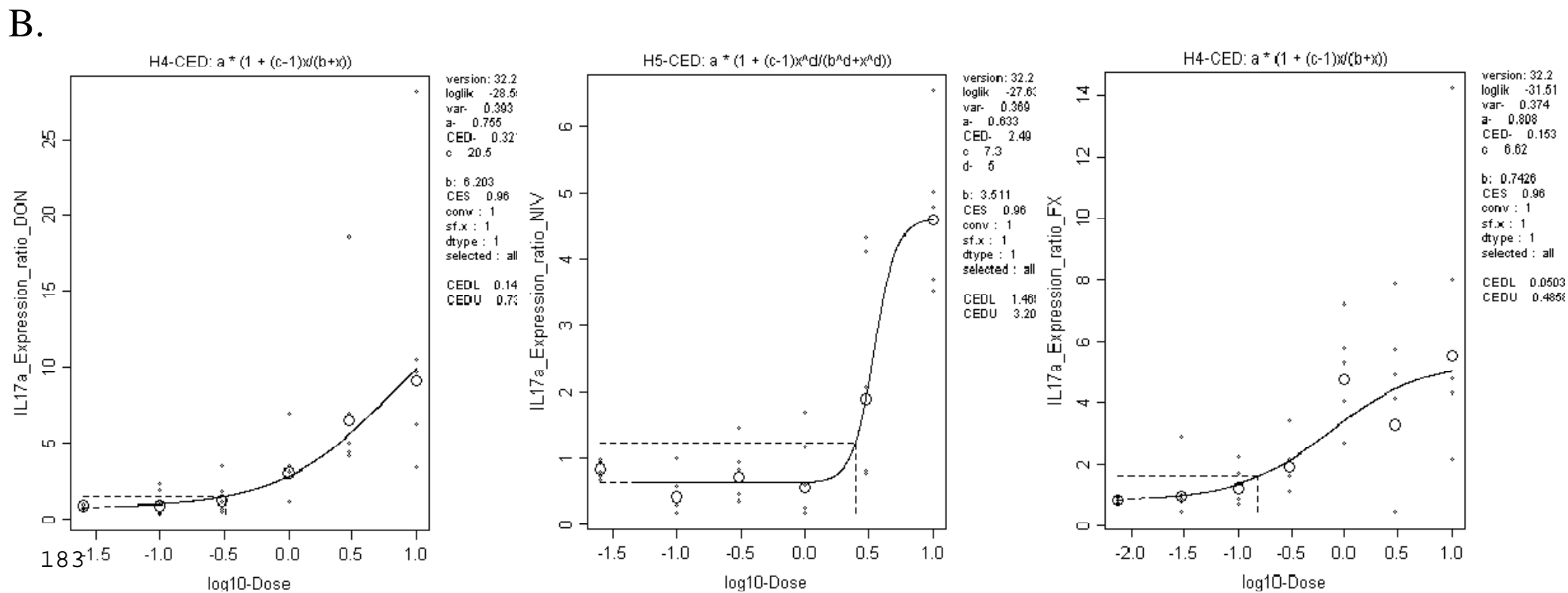
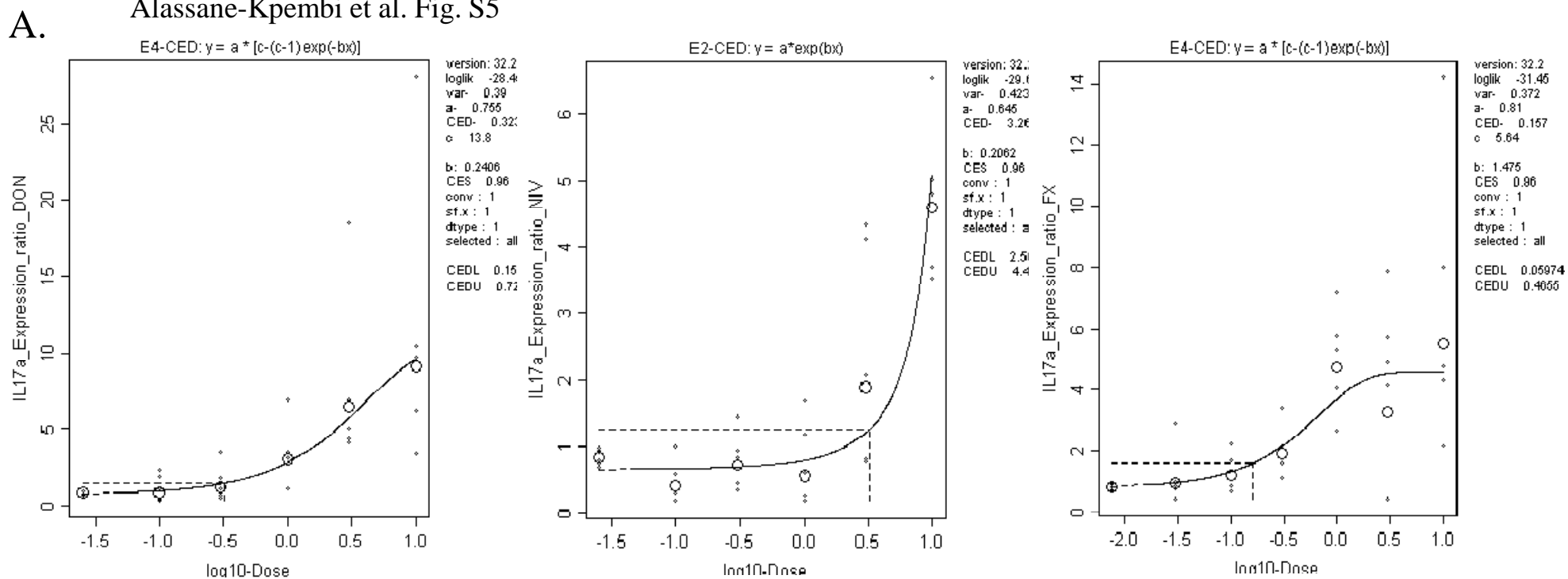


B.

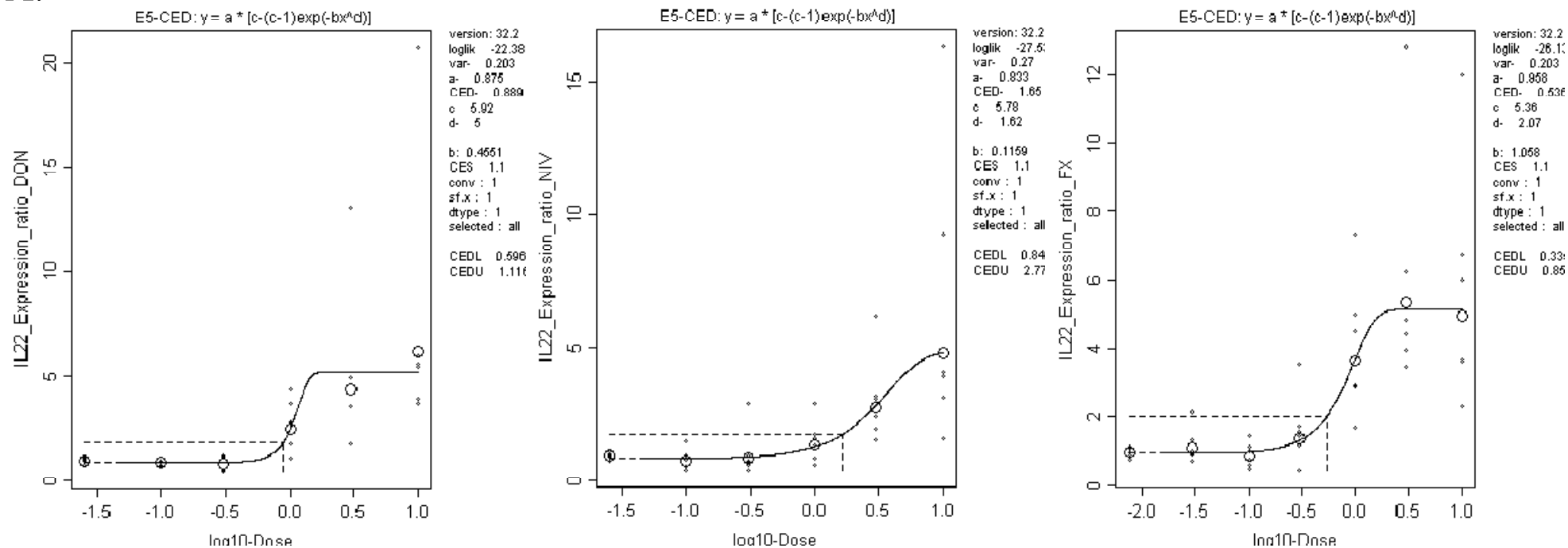


Alassane-Kpembi et al. Fig. S4

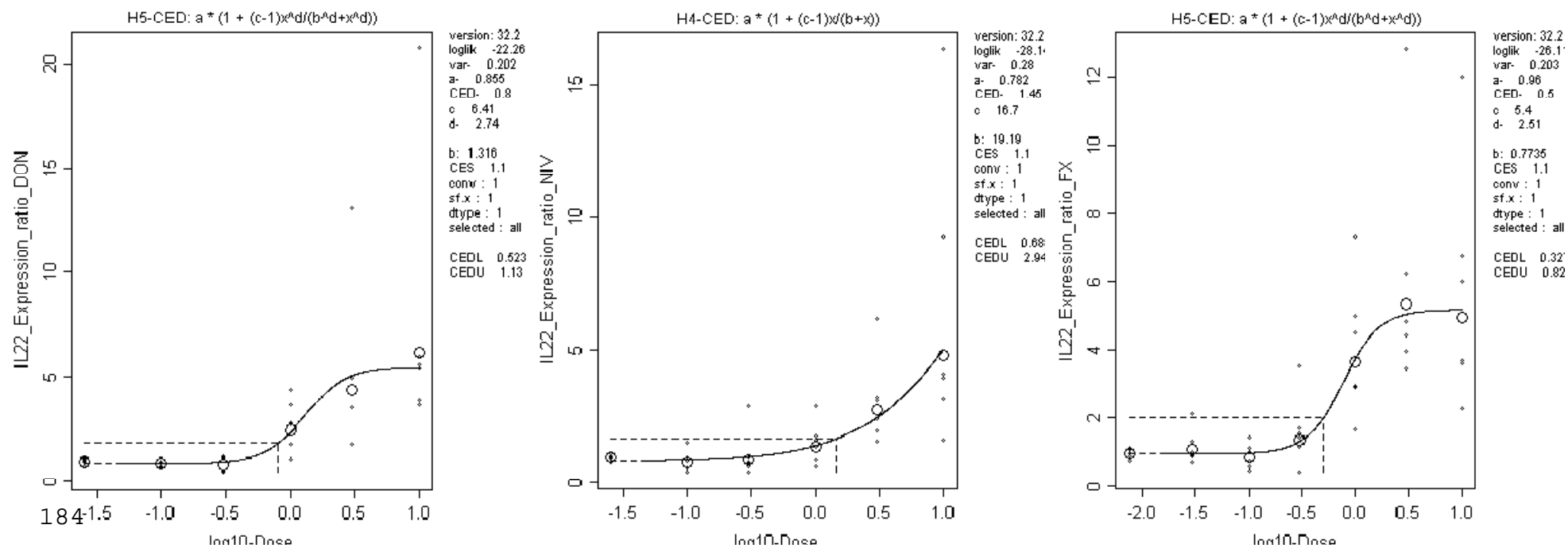




A.



B.



III. DISCUSSION GENERALE

1. L'intestin organe cible et modèle d'étude de la toxicité des TCTs B

La muqueuse intestinale représente la plus grande surface d'échange entre l'organisme et le milieu extérieur. A ce titre, elle peut être exposée dans des proportions importantes aux contaminants véhiculés par les aliments. Les mycotoxines constituent une classe de contaminants naturels très présents dans les aliments. On estime chaque année que 25% de la production agricole mondiale est contaminée (CAST, 2003).

Trois fonctions physiologiques essentielles de l'intestin sont particulièrement sensibles à la présence de contaminants dans les aliments (Turner, 2009; Rescigno, 2011). D'abord, la muqueuse intestinale représente une barrière physique entre le milieu intérieur et le contenu parfois hostile de la lumière intestinale. La muqueuse intestinale est également responsable de la digestion et de l'absorption des nutriments, ce qui suppose de la sélectivité dans la mise en œuvre de sa fonction de barrière. Et enfin, du fait de sa situation à l'interface entre le milieu interne et le contenu de la lumière intestinale, la muqueuse intestinale joue un rôle de métronome de la réponse immune de l'organisme face aux différents stimuli provenant des antigènes alimentaires et microbiens. Chacune de ces fonctions essentielles de l'intestin peut être ciblée par les TCTs B.

La fonction de barrière repose d'une part sur la continuité et le caractère cohésif de l'épithélium intestinal, et d'autre part sur la présence d'une double couche de mucus garantissant une séparation physique entre la flore intestinale et l'épithélium (Turner, 2009; Johansson *et al.*, 2011). Une abrasion survenant dans la phase mécanique de la digestion ou encore divers stimuli provenant de l'action de toxiques, de processus inflammatoires ou du stress oxydatif peuvent aboutir à une rupture de continuité de l'épithélium intestinal (Iizuka et Konno, 2011). Chez les mammifères adultes, l'épithélium intestinal est le tissu qui présente le pouvoir de régénération le plus important, le renouvellement complet de cet épithélium pouvant être accompli en 4 à 5 jours (Heath, 1996). Ce haut pouvoir de régénération garantit l'effectivité de la

fonction de barrière. Le DON exerce une activité cytotoxique qui peut perturber l'homéostasie du renouvellement de l'épithélium intestinal. Cette activité a été démontrée en utilisant différents modèles *in vitro* d'épithélium intestinal (Pinton *et al.*, 2009; Diesing *et al.*, 2011a; Vandenbroucke *et al.*, 2011). L'activité inhibitrice du NIV également a été démontrée dans un modèle murin de réparation de l'épithélium intestinal (Bianco *et al.*, 2012). A forte dose, le DON a été associé à la désintégration de la protéine de jonction serrée *zonula occludens-1* et à une réduction du niveau d'expression d'autres protéines de jonction serrées, notamment claudine 3 et claudine 4 (Pinton *et al.*, 2010; Diesing *et al.*, 2011b). Les protéines de jonction serrée jouent un rôle déterminant dans le maintien d'une étanchéité ou d'une sélectivité du passage des substances par la voie para-cellulaire (Tsukita *et al.*, 2001).

A la phase mécanique et enzymatique de la digestion fait suite une phase d'absorption des nutriments qui peut être la cible des effets des TCTs B. Il a pu être montré que le DON inhibait le transport intestinal du glucose avec une efficacité au moins aussi bonne que celle d'un inhibiteur pharmacologique, la phloridzine (Awad *et al.*, 2007). Le transport d'autres produits de la digestion, comme le palmitate et les mono-carboxylates, pourrait également être réduit en présence du DON aux concentrations usuellement rencontrées dans les aliments (Dietrich *et al.*, 2012).

La muqueuse intestinale module la réponse immunitaire de l'organisme face aux massives stimulations provenant aussi bien des antigènes alimentaires que de la flore microbienne du tube digestif. A cet effet, elle doit intégrer une multitude de signaux internes et externes pour coordonner la mise en œuvre adaptée et proportionnée d'une réponse immunitaire innée et/ou d'une réponse adaptative (Maldonado-Contreras et McCormick, 2011). Cette homéostasie est le résultat d'un dialogue entre les différentes lignées de cellules de l'intestin intervenant dans la réponse immune, et dont les chimiokines et les cytokines sont les médiateurs. L'exposition de la muqueuse intestinale au DON est associée à une dysrégulation de la réponse inflammatoire, et de plus en plus de travaux proposent l'hypothèse que cette mycotoxine à défaut d'être un

élément déterminant de l'étiologie multifactorielle de certaines maladies inflammatoires chroniques de l'intestin, pourrait en constituer un facteur aggravant (Maresca et Fantini, 2010; Moon, 2012; Cano *et al.*, 2013)

En utilisant un modèle Caco-2 dérivé de cellules d'adénocarcinome du colon humain, nous avons mené une étude comparative et une analyse des interactions toxicodynamiques des effets inhibiteurs du DON et du NIV et de leurs dérivés acétylés sur le renouvellement de l'épithélium intestinal. Le modèle Caco-2 a été largement utilisé dans les études de métabolisme, de transport et de toxicité des xénobiotiques (Artursson *et al.*, 2001; Boveri *et al.*, 2004). Cependant un certain nombre d'insuffisances rapportées pour ce modèle *in vitro* limitent les possibilités d'extrapolation des résultats aux réalités *in vivo*. En effet, l'origine tumorale de cette lignée lui conférerait un phénotype différent des cellules primaires de l'épithélium intestinal humain (Press et Di Grandi, 2008; Sun *et al.*, 2008). Caco-2 présenterait ainsi une inversion de son profil d'activités estérases se traduisant par des spécificités de métabolisation plus proches des hépatocytes que des entérocytes (Imai *et al.*, 2005; Ohura *et al.*, 2007). Les dérivés acétylés du DON et du NIV sont des substrats de carboxylestérases dont la toxicité est modulée par la présence et la position des radicaux acétyl (Wu *et al.*, 2010; Wu *et al.*, 2013). En conséquence, le modèle d'entérocyte Caco-2 pourrait paradoxalement mieux prédire la toxicité systémique des TCTs B, notamment ceux présentant des radicaux acétyl, et ne pas être adapté à la réalité de leur toxicité intestinale. **Nous avons pour cette raison réévalué la cytotoxicité intestinale individuelle et combinée des TCTs B en utilisant le modèle porcin d'épithélium intestinal IPEC-1.** Contrairement à la lignée Caco-2, IPEC-1 est une lignée entérocytaire non transformée qui a gardé toutes ses caractéristiques originelles d'épithélium intestinal, et qui du fait, permettrait une meilleure extrapolation *in vitro-in vivo* des résultats (Brosnahan et Brown, 2012; De Vos *et al.*, 2012). La lignée IPEC-1 a été utilisée dans plusieurs études relatives aux effets des contaminants alimentaires ou aux interactions entre la flore du tube digestif et la

muqueuse intestinale (Bouhet *et al.*, 2004; Bouhet *et al.*, 2006; Loiseau *et al.*, 2007; Koh *et al.*, 2008; Parthasarathy et Mansfield, 2009; Lucioli *et al.*, 2013).

Pour caractériser l'activité pro-inflammatoire des TCTs B sur la muqueuse intestinale, nous avons utilisé un modèle tissulaire *ex-vivo* d'explants de jéjunum de porc développé au sein de notre équipe (Kolf-Clauw *et al.*, 2009). Pour une étude d'immunotoxicité, ce modèle présente l'avantage de conserver l'architecture tissulaire nécessaire à la mise en œuvre du dialogue entre les différentes lignées cellulaires intervenant dans la réponse immune. Ce modèle a déjà été utilisé pour une analyse transcriptomique de l'activité pro-inflammatoire intestinale du DON, avec des résultats corroborés par une approche protéomique (ELISA) (Cano *et al.*, 2013). Les explants présentent également l'avantage de permettre de tester le grand nombre de conditions expérimentales inhérent aux études dose-réponse des TCTs B individuellement et en mélange. Ce modèle peut ainsi représenter une alternative intéressante aux études *in vivo* dans un contexte de réduction du nombre d'animaux enrôlés dans les expérimentations pour des considérations éthiques. Partant de six animaux, nous avons ainsi pu tester une trentaine de conditions expérimentales, alors qu'à puissance statistique égale, une étude *in vivo* aurait nécessité environ 200 animaux. La limite principale de cette approche *ex-vivo* reste la faiblesse de la variabilité biologique qu'elle permettrait d'appréhender.

Pour cette dernière partie de notre travail de thèse, le choix du modèle animal porcin nous a été dicté par le fait que par rapport à l'évaluation toxicologique des mycotoxines, cet animal peut être considéré comme une espèce modèle à double finalité pour la recherche agricole et la recherche biomédicale (Almond, 1996; Ireland *et al.*, 2008). Sa grande proximité de régime alimentaire et de physiologie digestive avec l'homme en fait un modèle de choix pour l'étude des effets des contaminants alimentaires sur la santé intestinale (Nejdfors *et al.*, 2000; Meurens *et al.*, 2012). Par ailleurs, cette espèce partage de fortes homologues de séquences génomiques avec

l'homme, ce qui facilite l'utilisation, au besoin, d'outils génomiques dérivés des banques de données humaines plus richement fournies.

2. Du renouvellement de l'épithélium intestinal comme marqueur de la toxicité des TCTs B

Les TCTs en général partagent les mêmes modes d'action (Larsen *et al.*, 2004). La toxicité aiguë pour ce groupe de mycotoxines de grande proximité structurale est ainsi caractérisée par un syndrome radiomimétique avec des effets communs comme la diarrhée, les vomissements, la leucocytose, les hémorragies, le choc circulatoire puis la mort. En toxicité chronique ces mycotoxines ont en partage l'anorexie, le retard de croissance, la baisse de l'efficacité alimentaire, les perturbations neuroendocrines, hématologiques et immunologiques.

Sur la base des données d'expositions collectées dans 17 pays européens, l'EFSA a estimé que le NIV ne représente pas un problème de santé publique (EFSA CONTAM, 2013). Dans le groupe d'âge des enfants de 12 à 36 mois qui constituent la catégorie de population la plus exposée à l'exception des végétariens, les limites inférieures et supérieures des expositions chroniques moyennes au NIV (12 – 484 ng /Kg poids corporel/jour) sont en effet bien inférieures à la dose journalière tolérable (DJT) établie à 1,2 µg/Kg p.c./jr. Cette DJT a été établie en partant des données brutes d'une étude portant sur l'exposition alimentaire sub-chronique des rats au NIV (Takahashi *et al.*, 2008). En considérant comme manifestation toxicologique critique la leucopénie observée, l'EFSA a dérivé une BMDL₀₅ de 0,35 mg/Kg p.c./j. A cette valeur a ensuite été appliqué un facteur d'incertitude de 300 prenant en compte les différences inter et intra-spécifiques et le défaut de données sur la toxicité chronique, la toxicité fœtale et la reprotoxicité (EFSA CONTAM, 2013). Il est à noter qu'en partant des données de la même étude pivot, d'une approche LOAEL (lowest observed adverse effect level), et l'application d'un facteur de sécurité de 1000 prenant en compte les différences inter

et intra-spécifiques et l'absence de NOAEL, la Food Safety Commission in Japan a préconisé une DJT plus faible, de 0,4 µg/Kg p.c./j (FSCJ, 2010).

La DJT fixée par le Comité conjoint FAO/OMS pour les Additifs Alimentaires (JECFA) pour le DON est de 1 µg/Kg p.c./j (JECFA, 2010). Cette DJT a été établie en appliquant un facteur d'incertitude de 100 à la NOAEL de 100 µg/Kg p.c./j mise en évidence par une étude consacrée au retard de croissance observé en toxicité chronique (2 ans) chez la souris. Partant ensuite du principe que le 3-ADON était converti *in vivo* en DON, et que de ce fait ce dérivé acétylé contribuerait à la toxicité globale du DON, le JECFA a proposé une DJT provisoire de groupe de 1 µg/Kg p.c./j pour le DON et ses dérivés acétylés (3-ADON et 15-ADON).

Contrairement aux dérivés acétylés du DON, aucune DJT n'a encore été fixée pour le dérivé acétylé du NIV, la FX.

La co-occurrence du DON, du NIV et de leurs dérivés acétylés dans les denrées alimentaires est attestée dans bon nombre de publications (Kim *et al.*, 1993; Eckard *et al.*, 2011; Schollenberger *et al.*, 2012). A des fins d'évaluation des risques, l'European Food Safety Authority (EFSA) préconise que la définition de valeurs toxicologiques de référence de groupe pour une classe de contaminants alimentaires devrait être envisagée dès lors que ceux-ci présentent un caractère de co-occurrence et partagent un même mode d'action (SCF, 2002). Cependant, en réponse à une requête de l'Union Européenne sur l'opportunité de définir une valeur de Dose Journalière Tolérable de groupe pour le DON, le NIV, et les toxines T-2 et HT-2, cette agence européenne de régulation a jugé que la masse des données scientifiques disponibles par rapport à la toxicité relative et à la nature des interactions toxicologiques des trichothécènes ne permettaient pas encore une telle évolution réglementaire (SCF, 2002).

Dans la première partie de nos travaux de thèse nous avons voulu proposer une échelle de toxicité relative pour le DON, le NIV et leurs dérivés acétylés vis-à-vis du renouvellement de l'épithélium intestinal, ainsi que la nature en termes de synergie,

d'antagonisme ou d'additivité de leurs effets combinés pouvant résulter d'une co-exposition alimentaire. **Sur des modèles d'épithélium dérivés de cellules d'adénocarcinome du colon humain et d'entérocytes non transformés de porcelet, nous avons pu montrer qu'à des doses de l'ordre du micromolaire correspondant aux niveaux d'exposition sur une base chronique révélées par des enquêtes alimentaires en Europe, ces TCT B pourraient affecter de façon significative l'homéostasie du renouvellement de l'épithélium intestinal. A ces doses biologiquement pertinentes, nous avons également pu observer que les TCTs B pourraient exercer une toxicité synergique vis-à-vis de l'épithélium intestinal.**

3. De la différence de toxicité relative des TCTs B sur les modèles Caco-2 et IPEC-1

En utilisant deux différents modèles *in vitro* de renouvellement de l'épithélium intestinal, nous avons voulu établir un référentiel toxique pour les TCTs B fréquemment véhiculés par l'alimentation. Sur les 2 modèles *in vitro*, la cytotoxicité observée était de l'ordre du micromolaire. En revanche si l'ordre croissant de toxicité (3-ADON – DON – 15-ADON – NIV – FX) était globalement le même dans les 2 modèles, des différences au niveau des écarts de toxicité entre ces cinq TCTs B ont été notées. Dans le modèle humain Caco-2, la FX était 20-30 fois plus toxique que le NIV alors que dans le modèle porcin IPEC-1 leurs toxicités étaient très voisines. De même le DON était 2 fois plus toxique que le 3-ADON dans le modèle Caco-2, alors que le rapport de toxicité était de l'ordre de 6 dans le modèle IPEC-1. Ces résultats traduisent plusieurs réalités récemment décrites (Abbas *et al.*, 2013; Wu *et al.*, 2013), à savoir :

- (i) Une acétylation sur l'hydroxyl en C3 s'accompagne d'une réduction constante mais d'intensité variable de la cytotoxicité des TCTs B pour les cellules de mammifères.

- (ii) Une acétylation de l'hydroxyl en C15 n'a que peu ou pas d'effet sur la toxicité des TCTs B
- (iii) L'acétylation de l'hydroxyl en C4 du NIV s'accompagne d'une augmentation plus ou moins marquée de sa toxicité pour les cellules de mammifères, selon la capacité à dé-acétyler de la lignée de cellules.

Les variations dans la modulation de la toxicité en fonction de la présence et de la position des radicaux acetyl sur le DON et le NIV que nous avons observées soulignent l'importance toxicologique des voies de métabolisation qui prennent en charge les TCTs acétylés.

Les dérivés acétylés des TCTs sont des substrats de différentes estérases, notamment les carboxylestérases (CESs) présentes dans les cellules des mammifères (Wu *et al.*, 2010). Ces CESs catalysent l'hydrolyse d'une grande variété de composés endogènes ou de xénobiotiques présentant une fonction ester ou amide, et jouent ainsi un rôle important dans l'activation ou l'inactivation de ces composés (Sato et Hosokawa, 1998). Les CESs des mammifères constituent une famille polygénique dont les isozymes sont regroupés en 4 principaux groupes et une multitude de sous-groupes. Les isozymes principalement exprimés dans le foie humain sont h-CE 1 et h-CE 2 appartenant respectivement au groupes CES 1 et CES 2, tandis que l'intestin exprime surtout l'isozyme h-CE 2 (Sato *et al.*, 2002). Des différences notables de substrats entre les deux isoformes sont connues. A titre d'exemple h-CE 2 hydrolyse l'acide acétyl-salicylique (aspirine) et la procaine, à l'opposé de h-CE 1 (Takai *et al.*, 1997). D'une façon générale, l'activité hydrolytique de h-CE 2 au niveau intestinal est connue pour altérer la perméabilité membranaire et le transport trans-épithélial des pro-drogues obtenues par acétylation des molécules actives parentales dans le but d'augmenter leur caractère lipophile (Taylor, 1996). A l'opposé de ce qui est observé au niveau intestinal, des travaux ont montré que les cellules Caco-2 présentent une inversion du profil d'expression des CESs, se traduisant par une prépondérance de l'activité estérase de type h-CE 1 identique à l'activité au niveau hépatique (Imai *et*

al., 2005; Ohura *et al.*, 2007; Ohura *et al.*, 2010). Dans notre démarche de constitution d'un référentiel toxique, les écarts de toxicité entre TCTs B (notamment entre acétylés et non-acétylés) tels qu'observés dans le modèle Caco-2 pourraient donc être assez éloignés des réalités *in vivo*.

Chez le porc, on note la même différence d'expression des isoformes, avec au niveau intestinal une expression de la Porcine intestinal carboxylesterase (PICE) et au niveau hépatique une expression de la Porcine Liver Esterase (PLE) (David *et al.*, 1998). h-CE 1, h-CE 2, PLE et PICE appartiennent à la superfamille des serine-estérases de 60-kDA, et en raison des fortes homologues de séquences entre l'homme et le porc, il n'est pas exclu que h-CE 2 et PICE soient structurellement très proches (Pindel *et al.*, 1997b; Pindel *et al.*, 1997a; Musidlowska-Persson and Bornscheuer, 2003). On peut penser que la lignée porcine non transformée d'entérocytes IPEC-1 qui a gardé les caractéristiques de son épithélium d'origine exprime PICE, et que les différences de toxicité relative des TCTs B dans les modèles Caco-2 et IPEC-1 sont la traduction des différences marquées de leurs profils d'activité estérase. En conséquence, le référentiel de toxicité tel qu'établi dans le modèle IPEC-1 pourrait être plus adapté à la réalité de la toxicité intestinale des TCTs B.

4. Les modèles d'étude *in vitro* des interactions toxicodynamiques

En toxicologie des mélanges, une interaction se définit comme un écart entre la toxicité réellement observée pour un mélange de composés toxiques et la toxicité prédite pour ce même mélange, en se basant sur les relations dose-réponse individuelles de ses constituants (Anand *et al.*, 2005). De façon pratique, évaluer une interaction dans un mélange de toxiques revient donc à dire si l'amplitude d'effet réellement observée pour ce mélange est supérieure (effet synergique), inférieure (effet antagoniste) ou égale (effet additif) à une amplitude d'effet théorique prédite sur la base d'un modèle d'additivité appliqué aux données de toxicité individuelle des constituants du mélange.

Trois modèles d'additivité ont classiquement servi de référence pour définir l'absence d'interaction toxicologique dans un mélange (Boedeker et Backhaus, 2010).

Le modèle d'additivité le plus usité dans les études *in vivo* ou *in vitro* analysant des interactions toxicologiques entre mycotoxines est le modèle d'addition des effets (Grenier et Oswald, 2011). Ce modèle est le plus intuitif et le plus simple de tous, et consiste à additionner les effets individuels connus des substances constituant le mélange. En l'absence d'interaction, l'effet d'un mélange de substances A et B serait ainsi égal à l'effet de la substance A plus l'effet de la substance B. Ce modèle d'additivité n'est envisageable que pour des substances présentant une évolution linéaire des effets en fonction des doses. Dans la pratique, le modèle d'addition des effets est d'une utilité très limitée, en raison de la nature habituellement sigmoïde ou hyperbolique des relations dose-réponse en toxicodynamie.

Pour des substances ayant les mêmes cibles et partageant un même mécanisme d'action, le modèle d'additivité de Loewe ou modèle d'addition des doses ou encore modèle d'addition des concentrations a été proposé. Il postule que les substances constituant le mélange se comportent comme des dilutions les unes des autres, et propose une relation dose-réponse intégrant une proportionnalité du paramètre de puissance (EC_{50} ou IC_{50}) et de la concentration de chacun des constituants dans le mélange. Les TCTs B objets de notre étude sont des époxydes de sesquiterpène qui ne diffèrent que par la présence ou l'absence d'un oxygène sous forme d'un hydroxyle, et par la localisation d'un radical acétyl. Cette proximité structurale pourrait suggérer une communauté de mécanismes d'action qui justifierait l'application du modèle d'additivité de Loewe. Toutefois, si les mécanismes d'action du DON commencent à être bien documentés ceux du NIV et, plus encore, ceux de leurs dérivés acétylés ont bénéficié de beaucoup moins d'attention (Pestka, 2010). De façon générale, les trichothécènes se lient à la sous-unité 60S du ribosome. Ils interfèrent avec l'action de la peptidyltransférase, perturbant ainsi la synthèse protéique en inhibant la phase d'initiation et/ou d'élongation. Ils sont ainsi classés selon la phase de synthèse

protéique où leur action est prépondérante en trichothécène de type I (initiation) ou de type E (élongation). Si l'appartenance du DON au groupe E est clairement établie, celle du NIV et de son dérivé acétylé, la FX est toujours discutée, en raison de leur action principalement sur la phase d'initiation, mais qui à forte dose se traduit plutôt par une inhibition de l'élongation (Ehrlich et Daigle, 1987).

Pour des substances ayant des mécanismes d'action différents et totalement indépendants, on définit un modèle d'additivité appelé Critère d'indépendance de Bliss ou modèle d'addition des réponses. Dans un mélange de substances aux mécanismes d'action totalement différents et strictement indépendants, cette indépendance se traduirait par le fait que l'effet relatif d'une substance reste inchangé en présence d'autres substances constituant le mélange. L'application de ce modèle nécessite elle aussi une connaissance exhaustive des mécanismes d'action des différentes substances contenues dans le mélange qui garantirait une absence de chevauchement de ces mécanismes. Ce qui n'est pas le cas dans le cadre des molécules étudiées dans cette thèse.

Des études d'interaction entre mycotoxines faisant appel à l'un et/ou l'autre de ces deux derniers modèles d'additivité existent (McKean *et al.*, 2006a; McKean *et al.*, 2006b; Tammer *et al.*, 2007; Mueller *et al.*, 2013). Toutefois aucun de ces modèles ne fait l'unanimité quand à sa plausibilité biologique (Goldoni et Johansson, 2007). On estime notamment que les substances toxiques ne pourraient avoir des mécanismes d'action totalement indépendants. Ainsi au niveau cellulaire par exemple les substances exercent leur toxicité en se liant aux macromolécules (ADN, ARN) ou à d'autres molécules d'intérêt biologique dont l'inactivation entraîne une cascade d'événements comme le stress oxydatif ou l'apoptose qui sont communs à bon nombre d'agents. Des chevauchements de mécanismes d'action des composés impliqués dans un mélange ne pourraient donc pas être exclus, ce qui introduirait un biais dans la définition de la réponse de référence pour l'analyse des interactions.

Pour analyser les interactions toxicologiques entre les TCTs B nous avons privilégié la méthode de Chou-Talalay qui est une approche qui ne formule aucune hypothèse sur les mécanismes d'action des substances constituant un mélange (Chou, 2006). En partant uniquement de la pente et du paramètre de puissance EC_{50} de chacune des mycotoxines et de leur mélange, il est possible de simuler, sur la base de la loi d'action de masse, l'amplitude de réponse toxicologique correspondant à la réponse théorique additive traduisant une absence d'interaction entre les différentes mycotoxines d'une combinaison donnée. La comparaison entre cette réponse théorique et la réponse observée expérimentalement pour le mélange de mycotoxines permet ensuite de tirer une conclusion par rapport à la nature éventuelle de l'interaction.

De façon générale, le défaut de quantification de l'amplitude des interactions a été identifié comme une faiblesse récurrente dans nombre d'études (Boobis *et al.*, 2011). L'intérêt de la méthode de Chou-Talalay est aussi lié au fait qu'elle permet une évaluation quantitative des interactions. Cette méthode propose deux indices pour appréhender quantitativement l'importance des interactions dans un mélange.

Le premier est le *Combination Index* (CI) dont la valeur quand elle est inférieure à 0.9 indique une synergie. Cet indice peut donc permettre de hiérarchiser des mélanges de toxiques en fonction des niveaux de synergie démontrés par les différents constituants. Cet index peut également éclairer sur la constance ou non de l'amplitude de synergie pour un même mélange en fonction du niveau d'effet présentant un intérêt biologique. **En analysant les valeurs de CI pour différents niveaux d'inhibition du renouvellement de l'épithélium intestinal par les TCTs B, nous avons pu évaluer la nature et l'amplitude des interactions sur une gamme de concentrations de mycotoxines, et notamment celles qui auraient une signification biologique pour le consommateur. Nous avons ainsi pu montrer que les amplitudes de synergie les plus élevées étaient observées aux faibles doses de combinaison de mycotoxines.** Ces doses correspondent aux niveaux d'exposition sur une base chronique révélés par

des enquêtes alimentaires en France et en Europe (Schothorst et van Egmond, 2004; Sirot *et al.*, 2013).

Le deuxième élément d'appréciation quantitative est le *Dose Reduction Index* (DRI). Dans une interaction synergique, la potentialisation mutuelle des effets des différents constituants du mélange se traduit par le fait qu'à égale amplitude d'effet, les doses de toxiques impliquées sont plus faibles. La notion de DRI a été introduite pour évaluer l'amplitude des réductions de doses de chacune des substances impliquées dans une combinaison synergique. Cette réduction sera d'autant plus prononcée que la synergie observée est forte. Dans le cadre de notre étude nous avons fixé comme seuil, le degré d'inhibition de renouvellement de l'épithélium intestinal associé aux niveaux d'exposition du consommateur connus pour les TCT B individuellement. **En se basant sur les valeurs de DRI observées pour les interactions synergiques, nous avons proposé pour les cas de co-exposition aux TCTs B les niveaux de présence de chaque mycotoxine qui ne se traduiraient pas par une augmentation du niveau d'effets délétères. En fonction du type de combinaison, les facteurs de correction ainsi proposés varieraient de 3 à 10.**

5. De la nature des interactions toxicodynamiques des TCTs B

L'analyse des interactions intervenant dans les combinaisons binaires et ternaires de TCTs B en utilisant les modèles IPEC-1 et Caco-2 de renouvellement de l'épithélium intestinal, montre d'une façon générale que le type et l'amplitude des interactions varient en fonction des niveaux d'exposition aux mycotoxines. Des synergies d'amplitude plus marquée aux faibles doses qu'aux fortes doses ont été notées pour toutes les associations entre le DON et ses dérivés acétylés, et pour les associations entre le DON et le NIV et entre le DON et la FX dans le modèle d'épithélium intestinal humain. La nature synergique de ces combinaisons de mycotoxines a été en partie confirmée dans le modèle d'épithélium intestinal porcin. A côté des différentes estérases cellulaires, l'implication de transporteurs

membranaires dans les mécanismes de toxicité des TCTs B pourrait être déterminante pour la nature des interactions résultant de leurs actions simultanées.

De par leur caractère amphiphatique, la présence de noyaux aromatiques et leur petite taille, les TCTs sont des molécules organiques qui présentent les caractéristiques structurales communes aux substrats du transporteur d'efflux P-glycoprotéine (Pg-P) (Schinkel et Jonker, 2003). L'implication de la Pg-P, mais également d'un autre transporteur d'efflux, la Multidrug Resistance-associated Protein 2 (MRP-2) dans les mécanismes de passage trans-membranaires et d'accumulation intracellulaire du DON et du NIV a été démontrée (Tep *et al.*, 2007; Videmann *et al.*, 2007). Par ailleurs, on a pu établir qu'une application du DON sur la bordure apicale des entérocytes qui présente les transporteurs Pg-P et MRP-2 entraînait une moindre toxicité que l'exposition par la voie baso-latérale (Takano *et al.*, 2006; Diesing *et al.*, 2011a; Diesing *et al.*, 2012). Cette toxicité accrue pourrait s'expliquer par le fait que les mécanismes d'efflux moins performants lors d'une application baso-latérale conduiraient à une plus grande accumulation intracellulaire de la mycotoxine.

En cas de présence intracellulaire simultanée de 2 TCTs, on peut envisager qu'il y ait une compétition pour les sites actifs des transporteurs, ce qui se traduirait par un efflux moins performant pour la molécule de moindre affinité. L'accumulation de la molécule ayant la toxicité intrinsèque la plus forte se traduirait ainsi par une interaction de type synergique du mélange, et l'accumulation de la molécule ayant la toxicité intrinsèque la plus faible par une interaction de type antagoniste.

Les phénomènes impliquant des mécanismes de transport actif sont par définition saturables. Ceci pourrait expliquer que les synergies ou les antagonismes observés étaient en général plus prononcées aux faibles doses.

En résumé, trois déterminants majeurs de la toxicité et du type des interactions entre TCTs B pourraient être proposés:

- (i) La présence et la position d'un radical acétyl qui augmenterait le caractère lipophile des TCT B et donc leur passage trans-membranaire (Poapolathep *et al.*, 2003; Poapolathep *et al.*, 2004)
- (ii) L'activité des carboxylestérases qui en hydrolysant à l'intérieur des cellules les dérivés acetylés entrainerait l'accumulation intracellulaire du DON ou du NIV et leur associations aux cibles déclenchant la réponse toxicologique
- (iii) La compétition entre TCTs B pour la fixation aux sites actifs des protéines d'efflux qui aboutirait à une accumulation intra-cellulaire des TCTs B de moindre affinité.

6. De l'activité pro-inflammatoire comme marqueur de la toxicité intestinale des TCTs B

Des études *in vitro* et *in vivo* montrent que l'exposition au DON provoque une réponse pro-inflammatoire. L'exposition orale de souris au DON s'accompagne ainsi d'une augmentation de l'expression de cytokines pro-inflammatoires et de chimiokines dans différents organes, notamment la rate, le foie, le rein et les poumons (Kinser *et al.*, 2004; Kinser *et al.*, 2005; Amuzie *et al.*, 2008; Pestka et Amuzie, 2008). L'augmentation de l'expression des cytokines pro-inflammatoires et des chimiokines a également été notée en exposant des modèles d'épithélium intestinal Caco-2 au DON (Maresca *et al.*, 2008; Van De Walle *et al.*, 2008). Toujours au niveau intestinal, cette augmentation de l'expression des cytokines pro-inflammatoires et des chimiokines a été confirmée en utilisant un modèle d'explants de muqueuse intestinale, et la complexité de ce modèle par rapport à un simple épithélium intestinal a permis de mettre en lumière un effet du DON sur la réponse adaptative pathogénique Th17 qui pourrait contribuer à renforcer son activité pro-inflammatoire intestinale (Cano *et al.*, 2013). Cet effet sur la réponse pathogénique Th17 va dans le sens de l'hypothèse selon laquelle, le DON, à défaut de jouer un rôle central dans l'étiologie multifactorielle des maladies inflammatoires chroniques de l'intestin (MICI), pourrait en constituer un facteur aggravant (Maresca et Fantini, 2010; Moon, 2012; Cano *et al.*, 2013; Maresca,

2013). En effet, à côté du paradigme Th1-Th2 (Mosmann *et al.*, 1986) qui a longtemps constitué le socle théorique de la réponse immunitaire adaptative, des données plus récentes suggèrent qu'au moins quatre sous-populations de lymphocytes T-auxiliaires (Th1, Th2, Th17 et Treg) pourraient orienter la réponse immunitaire adaptative dans un sens ou dans un autre (Liu *et al.*, 2012). La réponse Th17 notamment est mise en œuvre au cours de certaines infections par des bactéries extracellulaires ou des *Fungi*, ou pendant des processus liés à l'auto-immunité. Il s'agit d'une réponse de nature inflammatoire qui participe notamment au recrutement des neutrophiles vers le foyer inflammatoire (Stockinger et Veldhoen, 2007). Nous avons voulu à travers une étude dose-réponse caractériser l'activité pro-inflammatoire intestinale comme marqueur de la toxicité intestinale du DON et des TCTs B en général.

En utilisant le modèle explant de jéjunum de porc, nous avons observé que l'exposition de l'intestin aux TCTs B entraînait une activation dose-dépendante de l'expression des gènes codant pour la chimiokine IL-8 et les cytokines pro-inflammatoires IL-1 α , IL-1 β , et TNF- α . Les mécanismes d'action proposés pour l'activité pro-inflammatoire reposent sur les interférences du DON avec deux voies de signalisation cellulaire qui jouent un rôle déterminant dans la réponse inflammatoire. Il s'agit de la voie MAPKinase et de la voie NF- κ B.

En se fixant sur le ribosome, le DON, comme d'autres inhibiteurs de la synthèse protéique (α -sarcine, ricine, shigatoxines, anisomycine), initie une réponse au stress ribotoxique qui se traduit par l'activation des Mitogen-activated protein kinases (MAPKs) (Jordanov *et al.*, 1997; Zhou *et al.*, 2003). Ces MAPKs constituent une famille de protéines kinases qui interviennent dans nombre de processus cellulaires clés comme l'induction des gènes, la survie cellulaire ou l'apoptose, la prolifération et la différenciation, le stress cellulaire, et la réponse inflammatoire (Thalhamer *et al.*, 2008). Les MAPKs sont classés en 3 sous-familles: les Extra-cellular signal-regulated (ERK) 1/2, les c-Jun N-terminal kinases (JNKs), et p38 MAPK (Widmann *et al.*, 1999). La capacité du DON et d'autres TCTs à activer ERK, JNK et p38 a été

démontrée *in vitro* en utilisant des lymphocytes et différentes cellules de lignée myéloïde (Shifrin et Anderson, 1999; Yang *et al.*, 2000; Moon et Pestka, 2002; Chung *et al.*, 2003; Zhou et Pestka, 2003), et *in vivo* dans des rates de souris (Zhou *et al.*, 2003). Les voies ERK et p38 ont ainsi été associés à la trans-activation du médiateur de l'inflammation COX-2 et de la cytokine pro-inflammatoire TNF- α , alors que p38 contribuerait à la stabilisation des ARNm des cytokines pro-inflammatoires et des chimiokines (notamment IL-8) observées en présence du DON (Pestka, 2008).

En aval de l'activation de p38, il a pu être montré que l'action du DON et d'autres ribotoxines (le TCT macrocyclique satratoxine G, l'anisomycine, et la ricine) est reliée à une activation de la cathepsine L dans le cytosol entraînant un clivage en une multitude de fragments des ARN ribosomiaux 28S et 18S (He *et al.*, 2012a; He *et al.*, 2012b). A l'image des Damage-associated molecular patterns (DAMPS) qui initient une réponse immunitaire innée dans des processus non-infectieux, cette libération de fragments d'ARN dans le cytoplasme pourrait constituer, via la voie de signalisation NF- κ B, une deuxième porte d'entrée ou une étape d'amplification de la réponse inflammatoire induite par les ribotoxines (Newton et Dixit, 2012). Ainsi dans des modèles *in vitro* de culture de macrophages ou d'entérocytes, il est établi que l'induction DON-dépendante de la production de cytokines pro-inflammatoires était liée à une activation de la fixation sur son site nucléaire du facteur de transcription NF- κ B (Wong *et al.*, 2002; Maresca *et al.*, 2008; Van De Walle *et al.*, 2008).

L'inhibition de la re-synthèse de l'inhibiteur cytoplasmique I- κ B α par le DON est le mécanisme qui a été proposé pour expliquer la fixation accrue des protéines de la famille NF- κ B au noyau (Pestka *et al.*, 2004). Toutefois, le remaniement des stocks cellulaires des protéines NF- κ B p65, p52 et p50 observé en présence du DON suggère qu'en plus de l'inhibition de la re-synthèse d'I- κ B α , cette molécule pourrait également exercer une régulation au niveau transcriptionnel des stocks de protéines NF- κ B (Gray et Pestka, 2007). **Nous avons pu confirmer que l'exposition de l'intestin au DON,**

au NIV ou à la FX provoquait une activation dose-dépendante de l'expression du gène *NFκB 1*.

La mise en œuvre d'une réponse Th17 matérialisée par la surexpression des gènes codant pour l'IL-17A, l'IL-22 et l'IL-23A au niveau intestinal a été observée après une exposition au DON (Cano *et al.*, 2013). **Nos travaux ont confirmé cette réponse Th17 DON-dépendante au niveau intestinal, et l'ont étendue à d'autres TCTs B, le NIV et la FX.**

De petits fragments d'ARN du soi, générés par l'action de la RNase L sur l'ARN cellulaire, induisent une réponse immunitaire antivirale innée de type IFN- β , par des mécanismes de signalisation impliquant des Pattern recognition receptors (PRRs) bien spécifiques : Retinoic acid-inducible gene-1 (RIG-1) et Melanoma differentiation associated gene-5 (MDA-5) (Malathi *et al.*, 2007). RIG-1 et MDA-5 sont des senseurs nucléotidiques présents dans le cytosol qui interviennent dans la réponse contre divers virus à ARN, notamment les *paramyxovirus* et les virus *influenza* pour l'un, et les *picornavirus* et *norovirus* pour l'autre (Kato *et al.*, 2006; McCartney *et al.*, 2008). Il a été observé *in vitro* sur des cultures de macrophages que le DON et un autre TCT, la toxine T-2, pouvaient également entraîner la fragmentation de l'ARN ribosomal 28S en stimulant l'expression et l'activité de plusieurs RNases cellulaires dont la RNase L et la RNase 6 (Li and Pestka, 2008). La RNase 6 appartient à la famille des RNases A qui interviennent également dans des mécanismes de défense de l'hôte, notamment par la réduction du pouvoir infectieux des virus à ARN (Rosenberg, 2008). L'activation de la RNase L observée en présence du DON et de la toxine T-2 d'une part, et la fragmentation de l'ARN ribosomique constatée suite à l'effet du DON d'autre part, montrent clairement que les TCTs pourraient induire l'activation des récepteurs PRR. Cette hypothèse a d'ailleurs été explorée par Islam *et al.* (2006) qui ont toutefois conclu que le DON n'était pas un agoniste des Toll-like receptors (TLRs) 2, 3, 4, 5, 7, 8 et 9 humains. Les TLRs sont des récepteurs transmembranaires dont l'expression à l'intérieur de la cellule n'est associée qu'aux endosomes (Mogensen, 2009), alors que

la détection de fragments d'ARN ribosomal devrait incomber de façon prépondérante aux récepteurs PRR cytosoliques.

Il a été possible de déclencher une réponse Th17 avec des cellules Th-mémoires humaines, juste en activant des récepteurs PRR cytosoliques de type NOD2 sur des cellules dendritiques humaines par l'utilisation d'un agoniste spécifique, le muramyl dipeptide (MDP), un constituant du peptidoglycane de la paroi des bactéries gram + et gram- (Stetson et Medzhitov, 2007; van Beelen *et al.*, 2007). Un autre agoniste connu de NOD2 est l'ARN simple brin (ssRNA) cytosolique (Sabbah *et al.*, 2009).

Par un mécanisme similaire à celui de l'activation de NOD2, la réponse Th17 que nous avons observée, pourrait être liée à une stimulation des cellules Th-mémoires (CD4⁺) présentes dans la muqueuse intestinale (Mowat, 2003) par des cellules présentatrices de l'antigène dont les récepteurs PRR cytosoliques auraient été activés par des fragments d'ARN dans le cytoplasme provenant de l'action des TCTs B sur le ribosome.

Nous avons observé que l'exposition aux TCTs B entraînait une activation biphasique (courbe en cloche) aussi bien de l'expression génique de cytokines pro-inflammatoires et chimiokines de la réponse immunitaire innée que celle de cytokines de la réponse Th17. Une régulation non-monotonique de la production d'IL-8 par un épithélium de cellules intestinales Caco-2 exposé au DON a déjà été rapportée, avec un effondrement de la sécrétion de cette chimiokine pour des concentrations de mycotoxine supérieures à 25 µM (Maresca *et al.*, 2008). Une multitude de protéines caractérisées par des domaines SH2 ont été identifiées comme jouant un rôle clé dans les voies de signalisation cellulaire impliquant des cytokines notamment en inhibant par un système de rétrocontrôles négatifs la signalisation des cytokines inflammatoires pour juguler les altérations tissulaires qui pourraient résulter d'une activation importante et durable des voies de l'inflammation. Il s'agit des Suppressors of cytokine signaling (SOCS) dont l'expression et l'activité est tissu-

dépendante (Starr *et al.*, 1997). Une induction dose-dépendante de la production de plusieurs protéines SOCS a pu être observée dans le muscle et la rate de souris exposées oralement à des doses croissantes de DON (0,1-12,5mg/Kg p.c.) (Amuzie *et al.*, 2009). Un deuxième mécanisme de maintien du contrôle de la réponse inflammatoire dans les tissus est l'activation de la Mitogen-activated protein kinase phosphatase 1 (MKP1) qui aboutit à une déphosphorylation des MAPKs (Brondello *et al.*, 1999). Une surexpression du gène codant pour MKP1 a été observée dans la rate de souris, deux heures après leur exposition orale au DON à 25 mg/Kg p.c. (Kinser *et al.*, 2004). **L'effondrement de la réponse inflammatoire au niveau intestinal que nous avons observé pour des niveaux d'exposition à des TCTs B (DON, NIV et FX) supérieurs ou égaux à 10 µM, pourraient donc être associée à la mise en œuvre des SOCS et de MKP1.**

Tableau 1 : Evaluation du risque lié à l'exposition humaine au DON (adapté de Maresca, 2013)

Organe/système	Effet	Concentration requise	
Intestin	Inhibition de la prolifération cellulaire	1–5 µM	
	Surexpression des β-Défensines	2 µM	
	Réduction de l'absorption des nutriments	10 µM	
	Equivalent-concentration intestinale pour la DJT (JECFA)= 210 nM	Baisse de l'expression des mucines	10 µM
		Activation de la réponse pathogénique Th17	10 µM
		Hausse de la translocation bactérienne	10 µM
		Hausse de la production d'IL-8	1–20 µM
	Equivalent-concentration intestinale pour la fourchette haute d'exposition = 504 nM	Modification du microbiote intestinal	20 µM
Augmentation de la perméabilité intestinale		10–50 µM	
Cytotoxicité		>30 µM	
Baisse de la sécrétion d'IL-8		>30 µM	
Augmentation de la sécrétion d'IgA		500 µM	
Système immunitaire (circulation générale)	Augmentation de la prolifération lymphocytaire	1–30 nM	
	Activation des macrophages	1–100 nM	
Equivalent-concentration sanguine pour la DJT (JECFA) = 1,5 nM	Augmentation des cytokines pro-inflammatoires	0,1–1 µM	
	Baisse de la prolifération lymphocytaire	>100 nM	
	Inhibition des cellules NK	>150 nM	
Equivalent-concentration sanguine pour la fourchette haute d'exposition = 3,6 nM	Apoptose des macrophages	>300 nM	
	Apoptose des lymphocytes	10 µM	

Les concentrations requises sont celles qui sont reportées dans la littérature pour des altérations physiologiques. Les équivalents-concentration intestinale et sanguine sont calculés pour un homme adulte de 70 Kg avec un volume intestinal de 1L et un volume sanguin de 5L et en assumant les mêmes paramètres pharmacocinétiques pour le DON chez l'homme et le porc. DJT=dose journalière tolérable.

Une synthèse bibliographique des données de toxicité intestinale du DON indique que les concentrations de cette mycotoxine associées à des effets délétères sur l'intestin variaient de 1 μM à 500 μM (Maresca, 2013) (voir tableau 1). En rapportant cette gamme des concentrations potentiellement dangereuses à la concentration en DON attendue dans l'intestin d'un sujet adulte sur la base de la DJT provisoire proposée par le JECFA (0,21 μM) ou sur la base de la fourchette haute d'exposition du consommateur (0,5 μM), cette étude conclut que l'intestin semble globalement moins exposé au risque DON que le système immunitaire par voie générale. Les études expérimentales analysées dans cette synthèse bibliographique avaient en général privilégié une approche mécanistique par rapport à une caractérisation de la relation dose-effet de la toxicité du DON. En appliquant une approche Benchmark dose à des données de transcriptomique, nous avons voulu déceler de façon assez sensible les remaniements biologiques liés à l'exposition de la muqueuse intestinale aux TCTs. **Nous avons ainsi montré que des concentrations aussi faibles que 0,04 μM de FX ou 0,1 μM de DON et 0,1 μM de NIV perturbaient de façon significative l'homéostasie de la réponse inflammatoire de l'intestin exposé aux TCTs B.** Pour une exposition alimentaire aux mycotoxines, l'intestin est l'organe au niveau duquel la biodisponibilité est maximale. Notre étude montre que cet organe constitue une cible très sensible aux effets des TCTs, et que la toxicité intestinale des TCTs pourrait constituer un marqueur pertinent dans les procédures d'évaluation du risque.

IV. CONCLUSIONS - PERSPECTIVES

Le DON se classe actuellement en tête de peloton des mycotoxines les plus fréquemment rencontrées dans le monde, avec plus de 60% des échantillons d'aliment testés positifs. Cette mycotoxine est fréquemment retrouvée en association avec d'autres sur les substrats alimentaires, notamment d'autres TCTs B (3-ADON, 15-ADON, NIV, FX). La caractérisation des dangers associés à une exposition de l'organisme au DON peut être considérée à ce jour comme suffisamment avancée, ce qui n'est pas le cas des autres TCT B, et encore moins des effets associés aux co-expositions. Nous avons voulu caractériser au niveau de l'intestin, organe le plus exposé aux mycotoxines véhiculés par l'alimentation, la toxicité individuelle et les interactions toxicologiques des TCTs B.

Le renouvellement de l'épithélium intestinal et l'activité pro-inflammatoire au niveau intestinal se sont révélés être des marqueurs très sensibles dans le cadre de l'évaluation de la toxicité individuelle et de groupe des TCT B.

Nous avons montré qu'à des concentrations de l'ordre du micromolaire, les TCTs B inhibent le renouvellement de l'épithélium intestinal par ordre croissant de toxicité 3-ADON, DON, 15-ADON, NIV et Fus-X. Nos travaux ont également permis de caractériser l'activité pro-inflammatoire au niveau intestinal des TCT B, et l'analyse benchmark de données de transcriptomique a montré que l'exposition de l'intestin à des doses aussi faibles que 0,04 μ M de FX, 0,1 μ M de DON ou 0,1 μ M de NIV s'accompagne d'une activation significative des mécanismes de l'inflammation. Ces doses sont de l'ordre des concentrations attendues dans le chyle sur la base des valeurs toxicologiques de référence actuelles.

Notre analyse des interactions toxicologiques a révélé qu'aux faibles doses correspondant à des niveaux d'exposition rencontrés chez le consommateur français ou européen, les TCTs B pourraient inhiber de façon synergique le renouvellement de l'épithélium intestinal, avec des amplitudes de synergie d'un facteur 3 à 10. Partant du principe que les dérivés acétylés sont métaboliquement convertis en DON, le JECFA

(2010) a estimé qu'on pourrait considérer que les toxicités du DON et de ses dérivés acétylés sont équivalentes. Sur cette base, ce comité a recommandé une même DJT pour le DON seul et pour l'ensemble DON et dérivés acétylés. Nos résultats soulignent qu'en dépit des processus de conversion métabolique, les toxicités combinées du DON et de ses dérivés d'une part, et du DON, du NIV et de son dérivé acétylé d'autre part, pourraient ne pas être de nature simplement additive. Au niveau de l'épithélium intestinal où les voies impliquées dans le métabolisme des dérivés acétylés ne semblent pas prépondérants, nous avons observé qu'en fonction des TCTs impliqués, une interaction synergique ou antagoniste pouvait avoir lieu. Le mécanisme de ces interactions reste à élucider. Une interaction de type toxicocinétique, comme nous l'envisageons, impliquerait que la biodisponibilité par voie orale du DON selon qu'il est seul ou en mélange avec d'autres TCTs B pourrait ne pas être la même, et qu'une équivalence de toxicité entre le DON et ses dérivés ne pourrait pas se faire en ne prenant en compte que la seule conversion métabolique.

Nous poursuivons également l'analyse des interactions des TCTs B pour leur activité pro-inflammatoire au niveau intestinal. L'équation de l'effet médian de la loi d'action de masse utilisé pour les effets sur le renouvellement de l'épithélium intestinal a été dérivée pour des phénomènes biologiques d'inhibition. L'analyse des interactions dans les phénomènes d'activation comme l'effet pro-inflammatoire observé au niveau de l'intestin nécessite un ré-arrangement mathématique de cette équation (Stepkowski *et al.*, 1996; Chou, 2011), que nous avons initié en collaboration avec le Pr Chou.

A terme, la caractérisation de la nature et de l'amplitude des interactions relatives à la réponse pro-inflammatoire intestinale en cas d'une multi-exposition aux TCTs B permettrait une comparaison de la sensibilité des marqueurs d'effet "renouvellement de l'épithélium intestinal" et "inflammation intestinale" en ce sens qu'on aurait les gammes de doses effectrices pour chaque TCT B et pour chaque marqueur, et éventuellement l'intensité des synergies au niveau du marqueur.

La finalité de ces expérimentations *in vitro* et *ex vivo* reste de préfigurer le marqueur de toxicité intestinale, les gammes de doses et les combinaisons de TCTs B pertinents pour une caractérisation *in vivo* de la toxicité intestinale individuelle et combinée de ces mycotoxines. Cette approche présente l'avantage de permettre d'envisager une phase d'expérimentation *in vivo* la plus économe possible en animaux de laboratoire. Il est par exemple possible actuellement, par une technique de marquage au 5-Bromo-2'-deoxyuridine (BrdU), de mesurer *in vivo* la prolifération des entérocytes dans les glandes de Lieberkühn (Preidis *et al.*, 2012). Les indications fournies par les travaux de cette thèse sur les doses effectrices, et la nature des interactions en fonction des combinaisons de TCTs B et des doses d'exposition, pourraient permettre de réduire de façon substantielle le champ des investigations pendant la phase *in vivo*, si le renouvellement de l'épithélium intestinal était retenu comme marqueur le plus sensible de la toxicité intestinale des TCTs B.

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ANNEXE

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

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Abstract Enniatins, the most prevalent emerging mycotoxins, represent an emerging food safety issue, because of their common co-occurrence with other fusariotoxins such as trichothecenes co-produced by *Fusarium spp* on field grains and because of their extensive prevalence in grains. In this study, the intestinal toxicity of enniatin B1 (ENN) alone and mixed with the most toxic trichothecene T-2 toxin (T2) was characterized by using two biological models from pig, the most sensitive species: the intestinal cell line IPEC1 (in vitro exposure) and jejunal explants (ex vivo exposure). Dose-dependent decreases in cell proliferation in IPEC1 and in the histopathological scores of explants were observed for ENN at μM -levels and for T2 at nM-levels, with IC₅₀ values for ENN of 15.8 and 29.7 μM , and for T2 of 9.3 and 15.1 nM in vitro and ex vivo, respectively. Interaction analysis by probabilistic and by determinist

approaches showed a less than additive effect both in vitro and ex vivo, at IC₅₀ values, with increasing antagonism with decreasing concentrations of toxins. The results obtained by the determinist median-effect dose analysis and by the nonlinear regression analysis were concordant. All the median-effect doses estimated for IPEC cells were included in the IC₅₀ confidence intervals of the nonlinear regression fitting. Given the occurrence of enniatins, potential synergy following the co-occurrence of enniatins and the major fusariotoxins, especially trichothecene B deoxynivalenol should be investigated.

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

Introduction

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

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

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

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

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

Materials and methods

Toxins

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

In vitro assay: cell culture

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

Ex vivo assay: intestinal explant culture

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

containing 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 50 $\mu\text{g/mL}$ gentamicin and supplemented with D glucose (2.5 g/L) and 30 mM Alanine-Glutamine (Sigma) at 37 °C under CO₂-controlled atmosphere with orbital shaking. Uncultured control tissue was placed into fixative at the end of dissection time (0 h). Explants were exposed to purified T2 at 0, 0.3, 1, 3, 10 and 30 nM, or purified ENN at 0, 0.3, 1, 3, 10 and 30 μM , or to the mixture of T2:ENN at the same ratio as for cell culture (1:1,000) for 4 h. For histological analysis, jejunum explants were fixed in 10 % buffered formalin for 24 h. Histopathological and morphological assessment of the explants were carried out as previously described (Kolf-Clauw et al. 2009).

Data analysis: probabilistic and determinist approaches

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

Statistical analysis

Firstly, the individual dose–effect data were analysed to verify that both drugs produced effects that increased with dose. For each dose level, the percentage of proliferative cells compared to controls, and the explant scores are presented, as mean \pm SD of 5–6 independent experiments (from different animals for the intestinal explants). To test the individual and combined effect of the toxins according to the concentration, ANOVA analysis was used (Systat Software Inc, version 10, San Jose, USA) followed by Tukey and Dunnet tests if significantly different (*P* values \leq 0.05). The IC₅₀ values of each mycotoxin alone and of the mixture were calculated by extrapolating results from the concentration–response curves from each experiment, by nonlinear regression Hillslope modelling using the raw data (GraphPad Prism5 software Inc, San Diego, California, USA). To test the interactive effect, an isobologram approach was used, with the assumption of a constant potency ratio of ENN/T2, employing the concept of dose equivalence (Tallarida 2006). A constant potency ratio of 1,000:1 (ENN:T2) was used, and additive isobolograms represented by a line joining equally effective doses were constructed. The cytotoxic effect was identified as synergist, additive or antagonist according to the position of the combined effect related to the line of additivity (Borgert et al. 2001).

Median-effect doses and combination index

The second approach was based on a determinist analysis with the determination of combination index values (CI) by

the Chou-Talalay median-effect equation (Chou and Talalay 1984). The median-effect plot of Chou was applied to the individual toxins, and a median-proliferation dose (Dm) was calculated for each toxin (CompuSyn Software 2007) and compared with the IC₅₀ values calculated by the regression model. For the mixture of T2 and ENN, the Dm for the mixture was calculated. The combination index (CI) was calculated for a 10, 20 and 50 % inhibition of cell proliferation. CI is a quantitative measure of the degree of drug interaction of synergism and antagonism for a given endpoint of the effect measurement with CI < 1 indicating synergism, that is, a greater than expected additive effect and CI > 1 indicating antagonism, a smaller than expected additive effect.

Results

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

Each treatment, ENN (0.3–100 μM), T2 (0.3–100 nM) and ENN+ T-2 induced a dose-dependent decrease in intestinal epithelial cell proliferation (*P* < 0.001). ENN significantly decreased cell proliferation at all the concentrations tested (0.3–100 μM), with values ranging from 96.2 to 13.6 % proliferation when compared to control cells. T2 also decreased cell proliferation, from concentration of 3 nM and above, with relative cell proliferation of 80 % decreasing to 11.6 % at the highest concentration (Fig. 1). The IC₅₀ values were 15.80 μM (confidence interval of 13.02–19.17 μM) and 9.35 nM (confidence interval of 6.94–12.6 nM) for ENN and T2, respectively (Table 1). The binary mixture of T2 and ENN at the 1:1,000 ratio showed a dose-dependent effect on cell proliferation, ranging from 96.3 to 3.1 % compared to the control cells (Fig. 1), and an IC₅₀ of 14.41 μM (confidence interval of 10.55–19.67). The determination coefficients of the nonlinear regression Hillslope showed a better goodness of fit for isolated toxins than for the mixture, with values of 0.995 for enniatin, 0.989 for T2-toxin and 0.840 for the mixture (Table 1). The median-effect doses (Dm) calculated according to the determinist method were highly correlated to the IC₅₀ values, and the coefficients of correlation of the median-effect plot were between 0.925 and 0.997 (Table 1).

Individual and combined effects of the toxins on jejunal explant histopathology

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

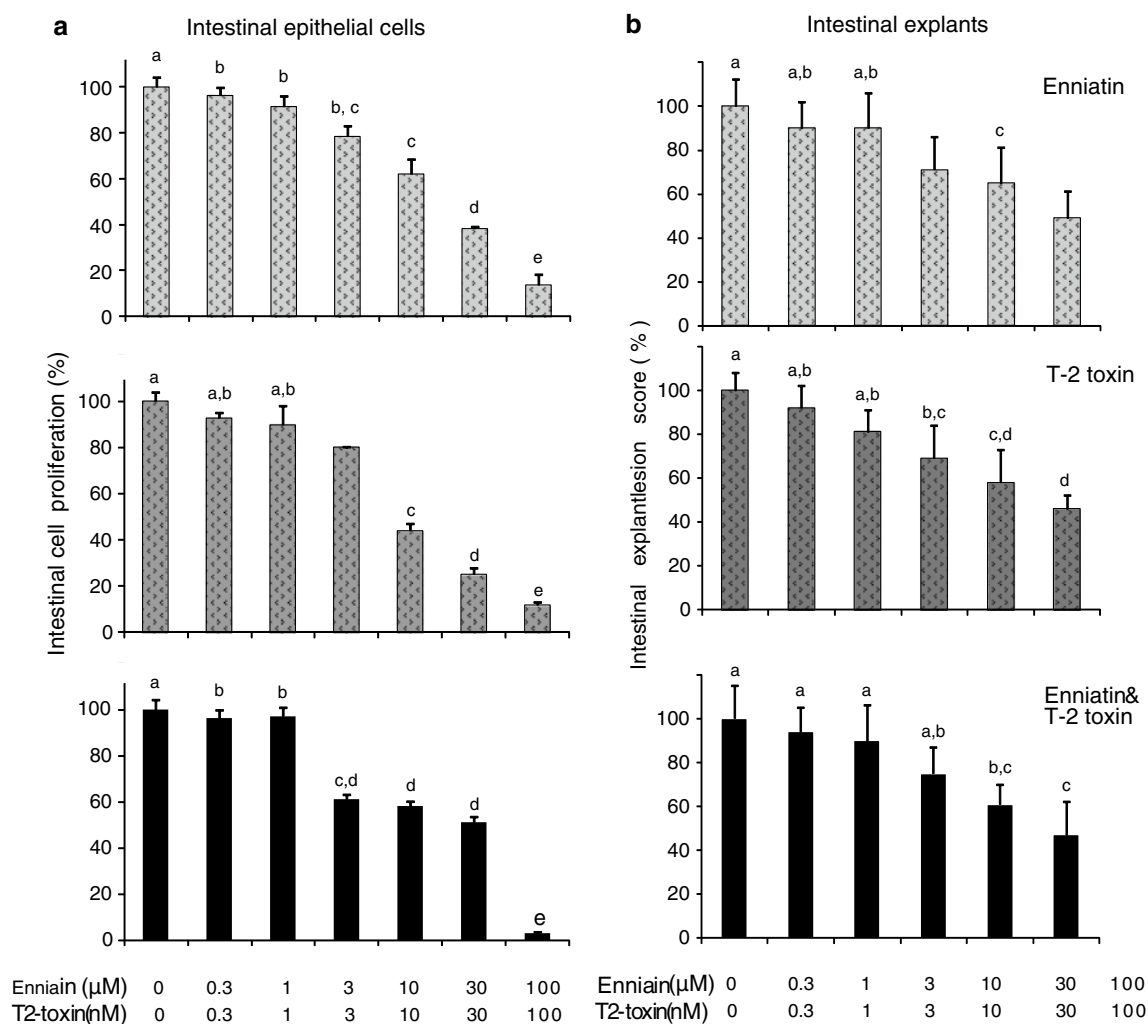


Fig. 1 a Effects of mycotoxins on the proliferation of IPEC cell cultures after 48-h exposure to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2-toxin 1,000:1). Mean relative proliferation compared to control cells (expressed as %) and the standard deviation (SD) of the mean. ANOVA analysis, bars without a common letters differ ($P < 0.05$). **b** Jejunal explants from 4 to 5 week-old piglets were exposed in vitro

for 4 h to different concentrations of enniatin B1 (ENN), T-2 toxin (T2) and their combination (ENN/T2 1,000:1) before histopathological examination and scoring assessment. For each concentration, 2–4 explants from the same animal were scored. Data are mean relative scores \pm SD from 6 different animals (expressed as %). ANOVA analysis, bars without a common letters differ ($P < 0.05$)

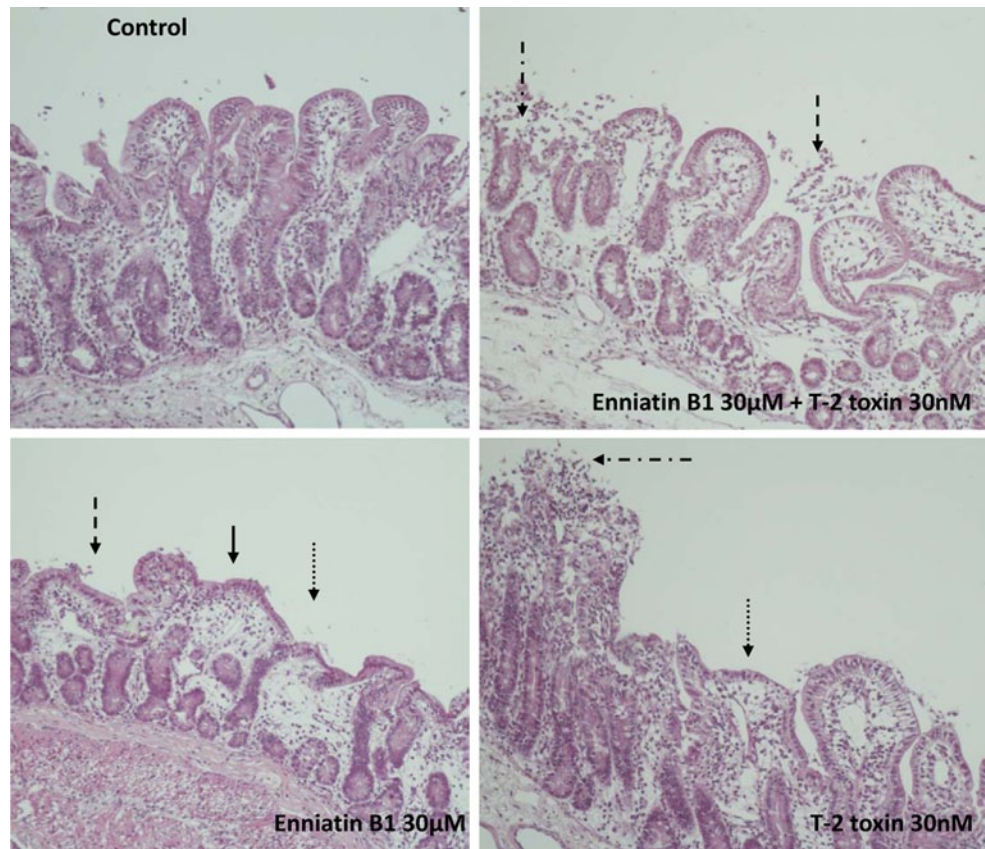
Table 1 IC50 and median-effect dose (Dm) in IPEC cell culture (Intestinal Porcine Epithelial Cell) and in porcine jejunal explants culture, following exposure to mycotoxins alone or in combination

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

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

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

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



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

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

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

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

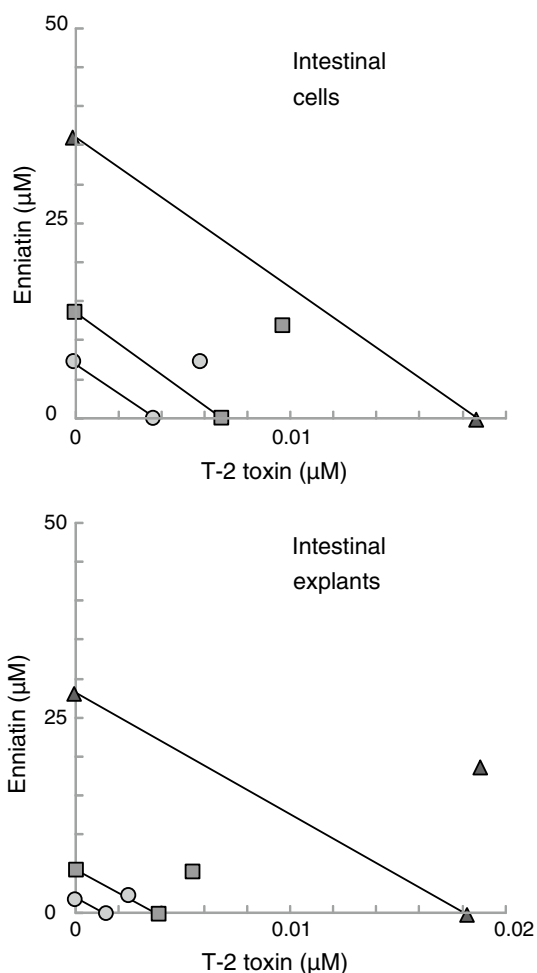


Fig. 3 Isobolograms illustrating the combined effect of the mixture of Enniatin B1 and T-2 toxin (1,000:1) for reaching 20 % ($F_a = 0.2$, filled circle), 30 % ($F_a = 0.3$, filled square), or 50 % inhibition ($F_a = 0.5$, filled triangle) of IPEC cell proliferation or of the scores of explants. The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

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

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

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

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

Discussion

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

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

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

Co-contaminations are frequent in cereals in Europe and worldwide (Rodrigues and Naehrer 2012) and studying combined toxicity of mycotoxins should help to protect consumers. The simultaneous appearance of T-2 toxin and enniatin B1 in the same samples can be inferred by the recent multi-mycotoxin analysis study of 83 feed samples (Streit et al. 2013). The chosen combinations were realistic, corresponding to some actual situations in food or feed, as T2 and ENN concentrations have been reported in the μ g/kg (Van der Fels-Klerx and Stratakou 2010) and in the mg/kg (Santini et al. 2012; Uhlig et al. 2006) ranges, respectively. In our study, two different analytical models were used that gave concordant results for the interaction. The T2 and ENN interaction was analysed according to a classical probabilistic approach, and also with a determinist method (Chou and Talalay 1984). This latter method provides a fundamental basis for assessing whether a combined effect is greater, equal or smaller than expected effect. This method was initially described for optimizing drug associations for pharmacologists (Chou 2006) and has subsequently been applied to toxicology studies of mycotoxins (Ruiz et al. 2011a, b). The present results from determinist analysis were correlated with the probabilistic results in the two biological models. For cell cytotoxicity, all the median-doses estimated by the determinist method

were included in the confidence intervals of Hillslope model, demonstrating a good concordance with the probabilistic approach. The Chou and Talalay method enables the nature of an interaction at various dose levels to be easily predicted. Interactions previously described between co-occurring mycotoxins showed either additive effects, antagonism or synergism, according to the biological model, the toxins, and the level of the effect. The combination of the two major trichothecenes T2 and DON was previously reported as antagonist in kidney cells, and similarly to our results, the strongest antagonism on cytotoxicity was observed at lower doses (Ruiz et al. 2011b). These latter authors used Chou and Talalay's method to analyse the fractional inhibition of proliferation by beauvericin, DON and T-2 toxin in combination. Strong antagonism was also observed with T-2 toxin and the emerging mycotoxin beauvericin, as for the IPEC cells and jejunal explants with ENN/T2 in our study. We observed stronger antagonism below 50 % relative proliferation inhibition in the two biological models, but interactions tending towards synergism at higher levels of the effects. Testing a combination at low concentrations appears highly relevant biologically compared to testing at high concentrations, such as above 75 % inhibition for cell relative proliferation as described previously (Ruiz et al. 2011b). We have to wonder about the relevance of the mechanisms underlying these interactions observed at high toxic concentration, whereas interactions at very low levels of effects are very relevant, because based on healthy cell metabolism and reactivity, allowing hypothesis relative to the mechanisms of these interactions.

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

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

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

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