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Differential impacts of individual and combined exposures of deoxynivalenol and zearalenone on the HepaRG human hepatic cell proteome

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ABSTRACT

Numerous surveys have highlighted the natural co-occurrence of deoxynivalenol (DON) and zearalenone (ZEA) mycotoxins in food and feed. Nevertheless, data regarding cellular mechanisms involved in response to their individual and simultaneous exposures are lacking. In this study, in order to analyze how low mycotoxin doses could impact cellular physiology and homeostasis, proteomic profiles of proliferating human hepatic cells (HepaRG) exposed for 1 h and 24 h to low DON and ZEA cytotoxicity levels (0.2 and 20 μM respectively), alone or in combination, were analyzed by LC-MS/MS. Proteome analyses of mycotoxin-treated cells identified 4000 proteins with about 1.4% and 3.7% of these proteins exhibiting a significantly modified abundance compared to controls after 1 h or 24 h, respectively. Analysis of the Gene Ontology biological process annotations showed that cell cycle, proliferation and/or development as well as on DNA metabolic processes were affected for most treatments. Overall, different proteins, and thus biological processes, were impacted depending on the considered mycotoxin and exposure duration. Finally, despite the important proteome changes observed following 24 h exposure to both mycotoxins, only the uptake of ZEA by the cells was suggested by the mycotoxin quantification in cell supernatants.

Biological significance: This study investigated the proteomic changes that occurred after DON and ZEA (individually and in combination) short exposures at low cytotoxicity levels in proliferating HepaRG cells using LC-MS/MS. The obtained results showed that the cellular response is time- and mycotoxin or mixture-dependent. In particular, after 1 h exposure, the DON + ZEA combination led to more proteomic changes than DON or ZEA alone, whereas the opposite was observed after 24 h. In addition, the significant cellular response to stress induced by ZEA after 24 h exposure seemed to be reduced when combined with DON. Thus, these results supported a possible mitigation by the hepatocytes when exposed to the mycotoxin mixture for a long duration. These findings represent an essential step to further explore adaptive cell response to mycotoxin exposure using with more complex incubation kinetics and combining different “omics” tools. Moreover, as mycotoxin quantification in cell supernatants showed different behaviors for DON and ZEA, this also raises the question about how mycotoxins actually trigger the cell response.

1. Introduction

The deoxynivalenol (DON) and zearalenone (ZEA) mycotoxins are both secondary metabolites produced by some *Fusarium* species, such as *F. culmorum*, *F. crookwellense* and *F. graminearum*, that can infect cereal crops under certain environmental conditions [1]. These mycotoxins are among the most widely distributed fusariotoxins in grains, respectively present in 59% and 45% of the cereal samples analyzed worldwide between 2009 and 2011 [2]. In addition, they are commonly found to co-occur in food commodities, mainly in the North Temperate Zone of the world [3].

DON, a type B trichothecene (TCT), is a polar organic compound

Abbreviations: α-ZAL, α-zearalanol; α-ZOL, α-zearalenol; β-ZAL, β-zearalanol; β-ZOL, β-zearalenol; DOM-1, de-epoxy deoxynivalenol; DON, deoxynivalenol; ESI, electrospray ionization; ER, endoplasmic reticulum; IC, inhibitory concentration; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MAPKs, mitogen-activated protein kinases; MS, mass spectrometer; MTS, mitrochondrial tetrazolium salt; QC, quality control; Q-TOF, quadrupole time-of-flight; TCT, trichothecenes; ZEA, zearalenone

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containing 3 free hydroxy groups (− OH) associated with its toxicity [4]. In several animal species, consumption of high doses of DON mainly causes acute temporary nausea, emesis, diarrhea, abdominal pain, headache, dizziness and fever, while chronic exposure to small doses elicits anorexia, growth retardation, impaired reproduction (reduced litter size) and adverse effects on the thymus, spleen, heart and liver [4–6]. A primary target of this mycotoxin is the innate immune system [7]. ZEA is classified as a non-steroidal estrogen. This fusariotoxin activates the estrogen gene and causes functional and morphological alterations on reproductive organs [8,9]. In particular, its exposure can lead to various estrogenic effects, such as decreased fertility, increased embryolethal resorption, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and change in serum levels of progesterone and estradiol [10]. Furthermore, ZEA is mainly metabolized in the liver, which also seems to be a main target for the toxin. While DON and ZEA are generally simultaneously present in food and feed, only few authors have studied their combined toxicological impacts in *in vitro* conditions. To date, data about the cellular mechanisms involved in the response to individual and combined exposures of DON and ZEA are still limited. This is likely because most of the studies use targeted analytical approaches and very few employ high-throughput, discovery-based methods (such as proteomics). The liver being our main detoxification organ, human hepatocytes represent one of the most relevant *in vitro* models for toxicity and cellular response studies.

In this study, we investigated the proteomic changes associated with DON and ZEA (individually and in combination) short exposures at low cytotoxicity levels in proliferating HepaRG cells, a cell line derived from a human hepatocellular carcinoma, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1. Mycotoxins

Mycotoxin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): DON (CAS#51481-10-8; purity > 98%) and ZEA (CAS#17924-92-4; purity > 99%). Standards were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) to final concentrations of 8×10^{-5} M for DON and 8×10^{-3} M for ZEA, and stored at − 20 °C.

2.2. Cell and culture conditions

Human hepatocytes (HepaRG) were acquired from Biopredic International (Saint Grégoire, France). They were cultured in William's E medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate at 37 °C with 100% relative humidity (RH) in a 5% CO_2 atmosphere. Culture medium was renewed every 2 to 3 days with fresh growth medium. The cells were passaged every 2 weeks at a density of 2.7×10^4 cells/cm^2 by a short time exposure (< 5 min) with a mixture of 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Sigma-Aldrich), and reseeded in a fresh medium.

For proteomic interrogations, biotriplicate samples of human hepatic cells were prepared by exposing 3×10^6 proliferating HepaRG cells, previously maintained two days at confluence in 25 cm^2 flasks, for 1 h and 24 h to DON (0.2 µM) and ZEA (20 µM) alone and in combination. The selected doses corresponded to 10% growth inhibition concentrations (IC_{10}) previously determined using a MTS cytotoxicity assay after 48 h exposure [11]. The final concentration of DMSO in cell cultures containing mycotoxins was maximum 0.5%. Appropriate control cultures without mycotoxin, but containing the same amount of solvent, were included as controls.

2.3. Proteomic sample preparation for tandem mass spectrometry analysis (LC-MS/MS)

Cellular preparations for proteomic analyses were completed as follows: cells were harvested and washed twice in ice-cold PBS by centrifugation at 400 × *g* for 10 min at 4 °C. Supernatants were removed and pellets were lysed in 100 µl of 50 mM ammonium bicarbonate with 8 M urea on ice. Protein concentrations were measured on each sample using the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Samples were diluted to yield a final concentration of 1 µg protein µl^{-1} and 100 µl of the obtained solutions were transferred to new tubes for digestion. Protein disulfide bonds were reduced with 6.6 µl of 1.5 M tris pH 8.8 and 2.5 µl of 200 mM tris (2-carboxyethyl) phosphine (37 °C, 1 h). For protein alkylation, 20 µl of 200 mM iodoacetamide was added and samples were then vortexed, and incubated for 1 h at room temperature in the dark. To absorb excess iodoacetamide, 20 µl of 200 mM dithiolthreitol was added, followed by vortexing and incubating at room temperature for 1 h. Samples were diluted with 800 µl of 25 mM ammonium bicarbonate and 200 µl of methanol, followed by digestion with Promega Trypsin (1:20; enzyme:protein) overnight at 37 °C. Samples were then evaporated on a SpeedVac to near dryness. Dilute formic acid (100 µl, 0.1%) was added to reduce the pH to < 2 and the samples were evaporated to dryness using a SpeedVac. Prior to mass spectrometry, samples were desalted using Microspin C18 columns following manufacturer's guidelines (Nest Group, Southborough, MA, USA). After desalting, the remaining solvent was evaporated with a SpeedVac. Finally, peptide samples were resuspended in 5% acetonitrile in 0.1% formic acid and frozen at − 80 °C until LC-MS/MS analyses were performed.

2.4. Mass spectrometry analyses

Samples were separated and introduced into the mass spectrometer (MS) by reverse-phase chromatography using a 30 cm long, 75 µm i.d., fused silica capillary column packed with C18 beads: Reprosil-Pur C18-AQ 3 µm (Dr. Maisch GmBH, Ammerbuch, Germany), and fitted with a 2 cm long, 100 µm i.d. precolumn (C18-AQ 3 µm Dr. Maisch GmBH). Peptides were eluted using an acidified (formic acid, 0.1% v/v) water-acetonitrile gradient (5–35% acetonitrile in 60 min). Mass spectrometry was performed on a Thermo Fisher (San Jose, CA) Q-Exactive (QE). The top 20 most intense ions were selected for MS2 acquisition from precursor ion scans of 400–1400 mz^{-1}. Quality control (QC) peptide mixtures (Pierce mixed peptide PRTC standards) were analyzed every 12th injection to monitor chromatography and MS sensitivity. Skyline was used to determine that QC standard retention time and isotopic distribution did not deviate > 10% through all analyses [12]. For quantitative analyses, biotriplicate samples from cells exposed to either DMSO, DON, ZEA or DON + ZEA were analyzed on the QE in technical triplicates using data-dependent acquisition (DDA), culminating in a total of nine analyses per treatment. Raw data have been deposited to the ProteomeXchange Consortium *via* the PRoteomics IDEntifications (PRIDE) [13] partner repository with the dataset identifiers PXD005840 (for 1 h exposure experiments) and PXD006267 (for 24 h exposure experiments).

2.5. Protein database searching and MS data interpretation

Following methods detailed by Nunn et al. [14], all tandem MS results were searched and interpreted with COMET v. 2016.01 rev. 2 [15,16], an open source tandem mass spectrometry (MS/MS) sequence database search engine for peptide identification. The protein database used for correlating spectra with protein identifications was a recent Human database consisting of 21,030 proteins (Human fasta 10-22-2015 from Swiss-prot database with isoforms at uniprot.org). Then, data were analyzed using the Trans-Proteomic Pipeline, which includes PeptideProphet and ProteinProphet [17], and then tabulated by

ABACUS, a computational tool for extracting and pre-processing spectral counts from MS/MS data sets, which aggregates data across replicates [18]. Only proteins with > 1 peptide and > 90% probability were retained for final data interpretations. The common method of spectral counting was selected to determine relative protein abundance. Spectral counting sums up the number of identified peptide tandem mass spectra resulting from a specific protein in order to estimate abundance of that protein relative to other proteins in the sample. Proteomics data were interrogated at the protein level using QSpec to determine relative quantities of proteins observed between control conditions and the different treatments (*i.e.* DON, ZEA and DON + ZEA) [19] (http://www.nesvilab.org/qspec.php/). QSpec was designed specifically for analyzing differential protein abundance data using label-free tandem mass spectrometry spectral counts. QSpec is reported using a fold change difference in abundance on a log base 2 scale. A reported positive fold change indicates a significant increase in abundance and a negative fold change indicates a significant decrease in abundance, while a reported fold change of zero indicates no significant difference between the treatments. Proteins were considered to be significantly increased or decreased in abundance if the reported Z score was $\geq |2|$ and the fold difference observed was $\geq |0.5|$. Then, the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) was used to identify significant biologically enriched processes within the large data set [20,21]. Proteins that were determined to be at significantly greater or lower abundance in response to each treatment condition were examined using this functional annotation tool. All 4000 proteins identified across biological and technical replicates were used as the background protein list.

2.6. DON and ZEA quantification in cell supernatants by LC-MS/QTOF

Supernatants from HepaRG cell cultures exposed 1 and 24 h to DON, ZEA and DON + ZEA mixture, as well as control cells, were collected and stored at − 20 °C until metabolite quantification. Metabolites were directly extracted from supernatants by dissolving in LC-MS grade acetonitrile (1:1), prior to filtration through a 0.2 µm PTFE membrane syringe 4 mm filter (GE Healthcare Life Sciences, Little Chalfont, UK) into an amber vial. A LC-MS/Quadrupole Time-of-Flight (Q-TOF) was used in order to separate and identify the extracted metabolites from each sample. The Agilent 1290 Series HPLC system included a binary pump and degasser, well plate autosampler with thermostat and a thermostat-capable column compartment. Two microliters of each sample were injected in the system and separation was achieved using a ZORBAX Extend-C18 column (2.1 × 50 mm and 1.8 µm, maximum pressure 600 bar) (Agilent, France) that was maintained at 35 °C throughout the chromatographic run. The flow rate was set to 0.3 ml min^{-1} using the following mobile phase: solvent A (milli-Q water + 0.1% formic acid + 10 mM ammonium formate) and solvent B (100% acetonitrile). Solvent B was maintained at 5% for the first 4 min, followed by a gradient of 5–100% of solvent B for 16 min, and then maintained at 100% during a 5-min post-time to equilibrate the column to original run conditions. Metabolites were detected using an Agilent 6530 Series Accurate-Mass Q-TOF mass spectrometer with an electrospray ionization (ESI) source operated in positive and negative ion modes. Mass spectrometer conditions were as follows: capillary voltage, 4 kV; source temperature, 325 °C; nebulizer pressure, 50 psig; drying gas, 12 l min^{-1} and ion range, 100–1000 *m/z*. LC-MS/QTOF calibrations were performed before each run following the mass spectrometer manufacturer's instructions. Relative DON and ZEA quantifications were carried out using the prepared mycotoxin standards previously described. For quantification, an 8 point linear range from 0.01 to 50 µM for both mycotoxins was prepared in acetonitrile. Some points were also prepared in the culture medium diluted in acetonitrile (1:2) and no matrix interference was observed. DON could be detected using the $[M\text{-}H]^+$ 297.133 *m/z* ion and $[M\text{-}Na]^+$ 319.115 *m/z* ion in ESI + mode while ZEA quantification was performed using the $[M\text{-}H^+]^-$ 317.139 *m/z* ion in ESI- mode. All metabolite characteristics used for LC-MS/QTOF analyses can be found in Table 1.

Table 1
LC-MS/QTOF method performance characteristics for mycotoxin quantification in supernatants.

Compound - formula	Retention time (min)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	R^2	ESI mode
DON – $C_{15}H_{20}O_6$	1.33	319.1151	297.1328	0.9925	+
ZEA – $C_{18}H_{22}O_5$	13.20	317.1392	n/a	0.9986	−

3. Results

3.1. HepaRG proteomic profiles following DON and/or ZEA exposures

To determine the mechanisms supporting the observed cytotoxicity of DON and ZEA in HepaRG cells [11], the proteomic profile changes induced by the individual and combined exposures to these two fusariotoxins after 1 h and 24 h were investigated by LC-MS/MS analysis. Proteomes of control cells as well as DON-, ZEA- and DON + ZEA-treated cells yielded the identification of 4000 inferred proteins with two or more unique peptides, representing about 19% of the human proteome (based on the human proteome from Swiss-prot database with isoforms (22-10-2017) consisting of 21,030 proteins).

After only 1 h treatment with DON, ZEA and DON + ZEA, the mixture DON + ZEA seemed to impact more proteins than single mycotoxins. Indeed, 21, 6 and 35 proteins exhibited a significantly enhanced or reduced abundance compared to control experiments, respectively (Z score $\geq |2|$ and observed fold difference $\geq |0.5|$) (Supplementary data, Table S1). Regarding the 21 proteins affected by DON, 7 were at higher abundances and 14 at lower abundances than in the control condition, while among the 6 proteins affected by ZEA, 5 were detected at higher levels and only 1 at a lower level than in the control. For the 35 DON + ZEA-response proteins, 6 exhibited a higher abundance and 29 a lower abundance compared to the control. The comparison of the proteomes of cells exposed 1 h to DON, ZEA and DON + ZEA showed that none of the identified proteins were common to DON- and ZEA-treated cells (and thus to DON-, ZEA- and DON + ZEA-treated cells), and very few were shared between cells co-exposed to DON + ZEA and cells exposed to DON and ZEA individually (Table 2 and Fig. 1A). More specifically, five DON-response proteins were also affected by the combination: the DNA topoisomerase 1 (P11387), DNA topoisomerase 2-alpha (P11388), DNA topoisomerase 2-beta (Q02880), RNA-binding protein 28 (Q9NW13) and tyrosine-protein kinase BAZ1B (Q9UIG0) and, for ZEA-response proteins, 2 proteins were also differentially abundant in DON + ZEA: the glyoxalase domain-containing protein 4 (Q9HC38) and carbamoyl-phosphate synthase [ammonia] (mitochondrial) (P31327), representing about 24% and 33% of the proteins that responded to DON and ZEA respectively (Table 2). In addition, the five identified proteins common to DON and DON + ZEA after 1 h exposure were at lower abundances than in the control whereas the two proteins common to ZEA and DON + ZEA were at higher abundances. Thus, a total of 55 proteins with a significant difference in abundances were identified from HepaRG cells after 1 h exposure with treatments. Among these 55 proteins, 39 were at a lower abundance than in the control treatment (representing 75%).

Regarding the 24 h exposure, ZEA treatment seemed to impact many more proteins than the DON or mixture conditions. After 24 h exposure to DON, ZEA and DON + ZEA 55, 96 and 39 proteins with a significantly modified abundance compared to the control treatment were identified, respectively (Supplementary data, Table S1). Concerning the 55 DON-response proteins, 5 were at higher abundances and 50 were at lower abundances while among the 96 proteins affected

Table 2
Common proteins to the different treatments (↗ = higher abundance proteins; ↘ = lower abundance proteins).

	DON 1 h					
ZEA 1 h	0 protein	ZEA 1 h				
DON + ZEA 1 h	5 proteins ↘: P11387, P11388, Q02880, Q9NW13, Q9UIG0	2 proteins ↗: Q9HC38, P31327	DON + ZEA 1 h			
DON 24 h	2 proteins ↘: P11387, P11388	1 protein ↘: P45973	2 proteins ↘: P11387, P11388	DON 24 h		
ZEA 24 h	3 proteins ↘: P11387, P11388, Q6P179	1 protein ↘: P45973	5 proteins ↘: P11387, P11388, Q13724, Q8NE71, P21695 (P21695 ↗ for DON + ZEA)	19 proteins ↘: A6NIZ1, O60271, P00505, P02795, P08183, P11233, P11234, P14678, P20073, P27487, P45973, Q8WWI1, Q969X5, P06756, P11387, P11388, P32321, P62834, Q86UP2	ZEA 24 h	
DON + ZEA 24 h	2 proteins ↘: P11387, P11388	0 protein	3 proteins ↘: Q8NE71, P11387, P11388	18 proteins: 2 ↗: P31350, Q99598 16 ↘: P01024, P02751, P0C0L4, P11717, P16401, P42166, P50402, Q07954, Q969S9, Q9BUJ2, P06756, P11387, P11388, P32321, P62834, Q86UP2	12 proteins ↘: P53582, Q13576, Q15418, Q6NT55, Q8N5N7, Q8NE71, P06756, P11387, P11388, P32321, P62834, Q86UP2	

by ZEA, 9 were detected at higher levels and 87 at lower levels compared to the control treatment. Similarly, for the mixture, most of the 39 response proteins exhibited lower abundances than in the control condition (4 with an enhanced abundance and 35 with a reduced abundance). Furthermore, 18 DON-response proteins and 12 ZEA-response proteins were also affected by DON + ZEA, which represented 33% and 13% of the DON- and ZEA-affected proteins, respectively (Table 2 and Fig. 1B). After this exposure duration, 6 proteins were common to all treatments, namely the deoxycytidylate deaminase (P32321), DNA topoisomerase 1 (P11387), DNA topoisomerase 2-alpha (P11388), integrin alpha-V (P06756), kinectin (Q86UP2) and Ras-related protein Rap-1A (P62834). These 6 proteins were all at a lower abundance than in the control. They represented about 4% of the 147 identified proteins from HepaRG cells displaying a significant difference in abundances (Table 2). Among these 147 proteins, 131 were at lower abundance (representing 89%), and all of those common to DON, ZEA and DON + ZEA were mostly detected at lower levels than in the control (Fig. 1B).

A comparison between 1 h and 24 h exposure showed that the number of proteins with a significantly altered abundance compared to the control highly increased in HepaRG cells after 24 h exposure with single mycotoxins, particularly with ZEA, while the number of impacted proteins remained stable with the mixture. Between the two treatment periods, we only observed two proteins in common for DON-treated cells (DNA topoisomerase 1 -P11387- and DNA topoisomerase 2-alpha -P11388-), and only one for ZEA-treated cells (chromobox protein homolog 5 -P45973-) (Table 2). In addition, the identified proteins common to the toxins alone and the combination after 1 h exposure were different to those shared after 24 h of exposure. Comparing the DON + ZEA-response proteins between 1 h and 24 h exposure, only three proteins were found in common: the ATP-binding cassette sub-family F member 1 (Q8NE71), DNA topoisomerase 1 (P11387) and DNA topoisomerase 2-alpha (P11388) (Table 2). All these common proteins were at lower abundances than in the control treatment after both incubation periods.

3.2. Molecular functions and subcellular locations of DON- and/or ZEA response proteins

The main molecular functions and subcellular locations of each differentially abundant protein induced by DON, ZEA or DON + ZEA exposure were categorized by searching the Uniprot (http://www.uniprot.org) and Gene Ontology (http://geneontology.org/) databases and are illustrated in Figs. 2 and 3. It is important to note that the same protein can possess several molecular functions and can be localized in different subcellular parts.

Molecular function annotations of the mycotoxin-response proteins (Fig. 2) revealed that, after 1 h exposure, all 21 DON-response proteins had binding and/or catalytic activity. More specifically, proteins with a binding molecular function mostly targeted DNA, RNA and proteins (80%), while 60% of those with a catalytic activity were hydrolases. In addition, the subcellular location analysis (Fig. 3) showed that almost half of these 21 DON-affected proteins were cytoplasmic and about 43% were localized in the nucleus. For the 6 ZEA-response proteins, they all showed either a binding (67%) or a catalytic activity (33%), all with a different binding target or a different catalytic function. Moreover, they were primarily localized in the nucleus (50%) and to the mitochondrion (33%). Similarly to DON- and ZEA-response proteins, most of the 35 DON + ZEA-targeted proteins after 1 h exposure were annotated with a binding and/or a catalytic activity (> 90%). More specifically, > 60% of the proteins with a binding activity targeted DNA or RNA and almost 60% of those with a catalytic activity had a hydrolase activity. Furthermore, > 60% of the DON + ZEA-affected proteins were localized in the nucleus and 25% were cytoplasmic.

After 24 h exposure, the analysis of the molecular functions (Fig. 2) further showed that a large majority of the 55 DON-response proteins

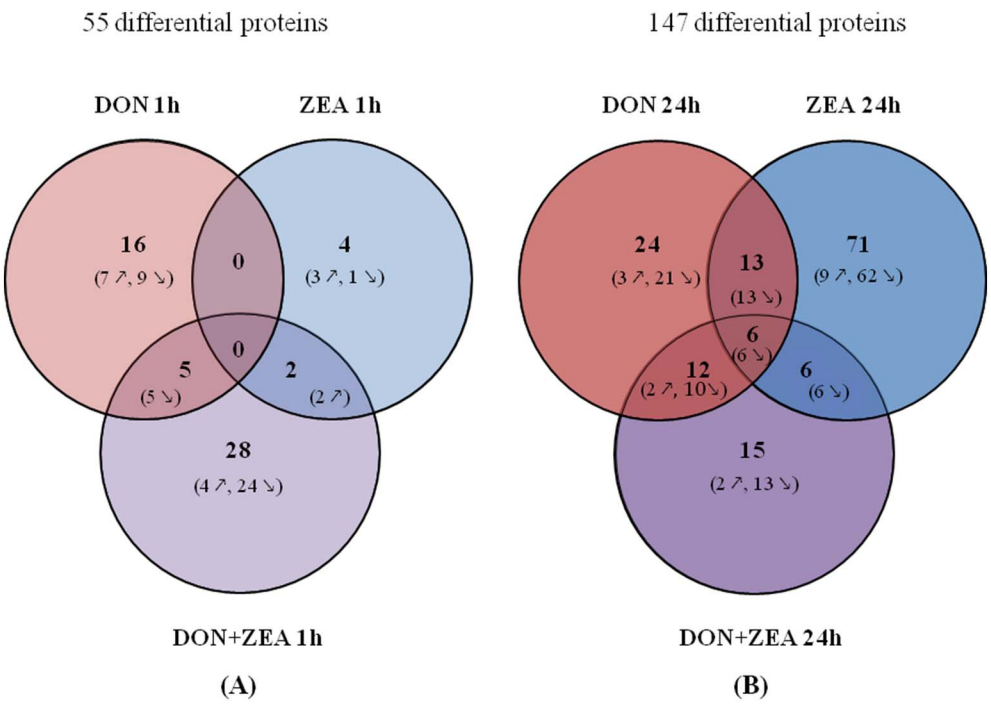

Fig. 1. Venn diagrams of differentially abundant proteins in HepaRG cells after (A) 1 h and (B) 24 h treatment with DON and/or ZEA. Bold numbers represent the number of proteins exhibiting differential abundance compared to the control. Numbers for proteins exhibiting higher (↗) and lower (↘) abundances are also provided.

(about 80%) had a binding and/or a catalytic activity. In particular, most of those with a binding activity targeted nucleic acids (41%) and proteins (24%), while those with a catalytic activity mainly belonged to the hydrolase family (about 50%). In addition, the analysis of the subcellular locations (Fig. 3) showed that 38% of the DON-affected proteins were nucleic, 36% were cytoplasmic and 22% were localized in the cell membrane. Concerning the 96 ZEA-response proteins after 24 h exposure, they mostly presented a binding or a catalytic activity (74%) with several proteins exhibiting a nucleic acid or protein binding activity (38%) or a hydrolase activity (55%). Moreover, 40% of these 96 targeted proteins were localized in the nucleus, the same proportion was cytoplasmic and only 18% were mitochondrial. Regarding the 39 DON + ZEA-affected proteins, most of them had binding and/or catalytic activity (70%) after 24 h exposure. > 60% of the proteins had a binding activity targeting DNA or RNA and almost 60% of those with catalytic activity were hydrolases. Furthermore, after 24 h exposure, 31% of the DON + ZEA-response proteins were localized in the nucleus, 31% in the endoplasmic reticulum (ER) and 28% in the cytoplasm.

The molecular function analysis (Fig. 2) revealed that the remainder

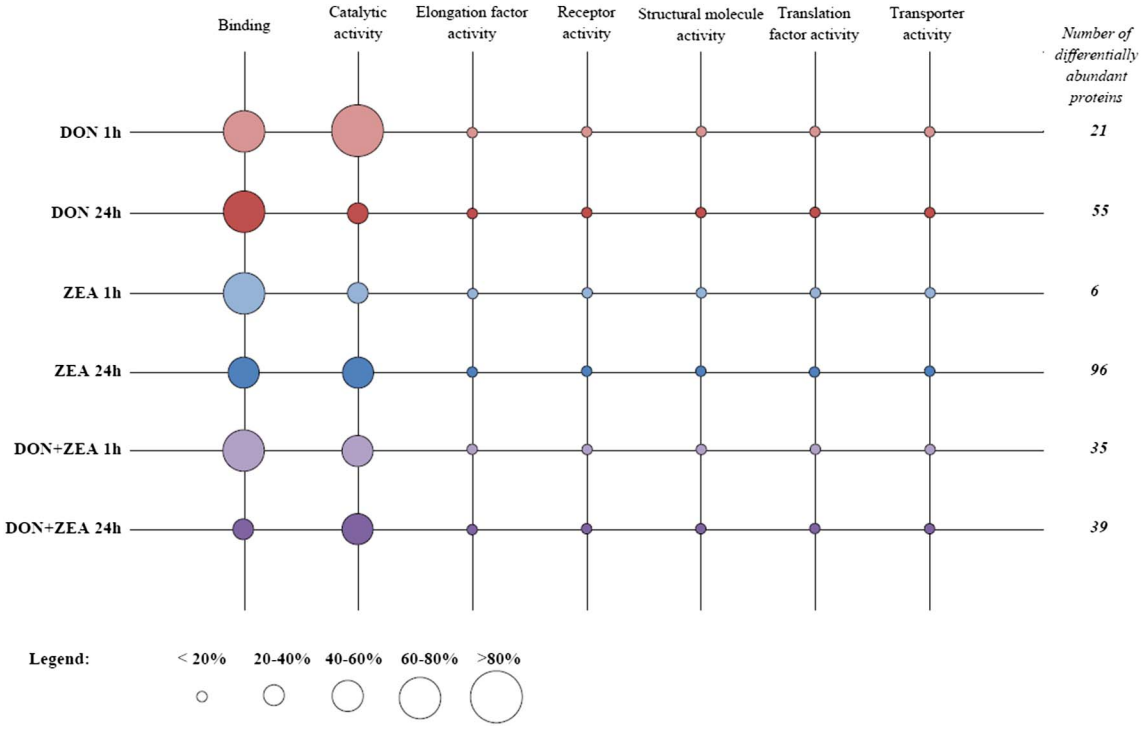

Fig. 2. Annotated molecular functions of the response proteins in HepaRG cells after 1 h and 24 h exposure to DON, ZEA and DON + ZEA. Results are expressed as the percentage of affected proteins with the considered molecular function among all the affected proteins for each treatment.

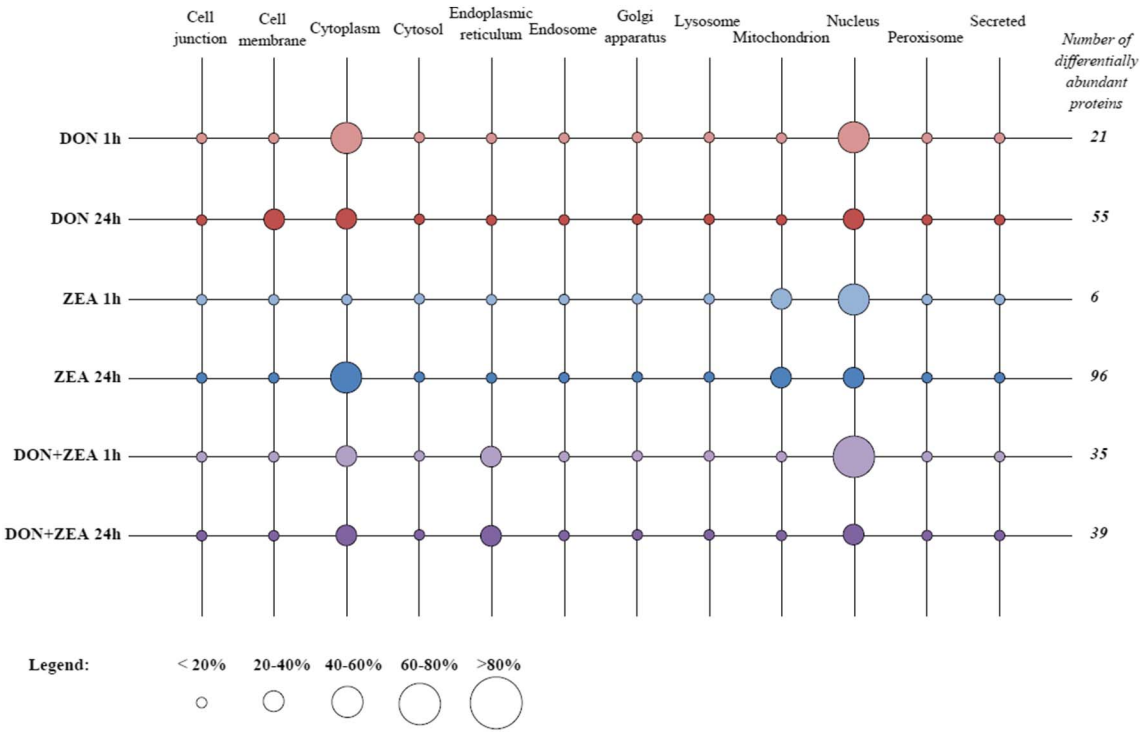

Fig. 3. Annotated subcellular locations of the response proteins in HepaRG cells after 1 h and 24 h exposure to DON, ZEA and DON + ZEA. Results are expressed as the percentage of affected proteins with the considered subcellular location among all the affected proteins for each treatment.

of the mycotoxin-response proteins after both 1 and 24 h exposures had elongation factor, receptor, structural, transporter and/or translation regulator activity. Based on the definition of "catalytic activity" given by the Gene Ontology database, these first results indicated that the DON and ZEA mycotoxins primarily affected enzymes that targeted nucleic acids and proteins in HepaRG cells. Furthermore, for all treatments, the analysis of the subcellular location annotations revealed that a minor part of the affected proteins by these mycotoxins, alone or in mixture, were localized in organelles such as Golgi apparatus, endosome, peroxisome and lysosome, or to the cell junction and some others were secreted by the cells. The results suggested that DON might induce nucleic and cytoplasmic changes in human hepatocytes, while ZEA might also induce mitochondrial changes. When present together, these two mycotoxins mostly targeted the nucleus after 1 h exposure as well as the ER after 24 h exposure.

3.3. Analysis of the biological process annotations of DON- and/or ZEA response proteins

Study of the mycotoxin-response proteins with the functional annotation tool DAVID provided information about the main biological processes affected by DON and ZEA alone and in combination in human hepatic cells. Only biological process categories containing at least two proteins and with a P value < 0.05 were retained (Supplementary data, Table S2).

After 1 h exposure, DON induced significant abundance changes for proteins involved in 9 biological processes, the most represented ones being cell division, chromosome segregation and protein modification by small protein conjugation, with 5 proteins involved in each (corresponding to 24% of the 21 DON-response proteins). Regarding the 6 ZEA affected proteins after 1 h exposure, no enriched biological processes were identified by DAVID. For the DON + ZEA condition, more biological processes were affected than DON and ZEA alone ($n = 27$) and targeted chromosome organization, DNA metabolic process and cell cycle involving 13 (37% of response proteins), 12 (34%) and 10 (31%) proteins, respectively. In addition, 6 biological processes constituted a common response between differentially abundant proteins after 1 h exposure to DON and 1 h exposure to DON + ZEA (chromosome segregation, DNA topological change, embryonic cleavage, meiotic chromosome separation, protein sumoylation (*i.e.* attachment of a small ubiquitin-like modifier) and resolution of meiotic recombination intermediates), with the same proteins contributing in each case.

After 24 h exposure, 47 biological processes appeared to be affected by the DON exposure. The most represented ones were involved in transport and adhesion, such as vesicle-mediated transport ($n = 17$ proteins), cell adhesion ($n = 14$), biological adhesion ($n = 14$) and transport regulation ($n = 13$) (corresponding to 31%, 25% and 24% of the 55 DON-regulated proteins, respectively). In addition, 11 proteins (*i.e.* 20%) were also involved in cell development and cell proliferation. Regarding ZEA, 30 biological processes appeared to be affected, the most important ones being cellular metabolic process ($n = 25$ proteins), cellular response to stress ($n = 22$) and cell proliferation ($n = 16$) (representing 26%, 23% and 17% of the 96 ZEA-regulated proteins respectively). Moreover, several biological processes involved in the cellular response to numerous compounds including drugs seemed to be impacted following 24 h exposure to ZEA (about 10 biological processes from 21 proteins). For the DON + ZEA mixture, after 24 h exposure, 31 biological processes were identified. Most of the DON + ZEA-response proteins after 24 h exposure were involved in gene expression (n = 16) and hydrolase activity ($n = 10$) regulations (corresponding to 41% and 26% of the 39 DON + ZEA-regulated proteins respectively). Furthermore, after 24 h exposure, 12 biological processes were common to DON and DON + ZEA (apoptotic cell clearance, regulation of apoptotic cell clearance, positive regulation of apoptotic cell clearance, blood vessel development, blood vessel morphogenesis, extracellular matrix organization, extracellular structure organization, phagocytosis, regulation of phagocytosis, positive regulation of phagocytosis, positive regulation of endocytosis, and positive regulation of transport) while only 1 biological process (phagocytosis) was common to ZEA and DON + ZEA (which was common to DON too).

Table 3
Measured concentrations of DON and ZEA (μM) in the cell supernatants after 0 h, 1 h and 24 h of exposure with HepaRG cells (mean ± SD of concentrations from 3 independent experiments quantified using LC-MS). * = Mean of the final concentration significantly different from the initial concentration ($P < 0.05$); N.D. = not detectable.

Culture condition	Control culture	DON-treated culture	ZEA-treated culture	DON + ZEA-treated culture
Initial concentration of DON (μM)	ND	0.20 ± 0.0	ND	0.20 ± 0.0
Final concentration of DON after 1 h exposure (μM)	ND	0.26 ± 0.0	ND	0.23 ± 0.1
Final concentration of DON after 24 h exposure (μM)	ND	0.27 ± 0.4	ND	0.24 ± 0.4
Initial concentration of ZEA (μM)	ND	ND	20.0 ± 0.0	20.0 ± 0.0
Final concentration of ZEA after 1 h exposure (μM)	ND	ND	21.4 ± 0.6	20.2 ± 0.8
Final concentration of ZEA after 24 h exposure (μM)	ND	ND	0.03 ± 0*	0.06 ± 0*

A comparison of the affected biological processes after 1 h and 24 h of treatment showed that, for DON, none were common between both exposure durations while, regarding the mycotoxin combination, only embryonic cleavage was in common.

3.4. Measurement of DON and ZEA concentrations in cell supernatants after 1 h and 24 h exposure

In order to quantitatively evaluate the DON and ZEA uptake by the HepaRG cells after 1 h and 24 h exposures, concentrations of these two fusariotoxins were determined in cell supernatants at the end of the incubation periods and compared to the initial concentration used (namely IC_{10}). Based on the standard curves (linearity (R^2) > 0.99; data not shown), mycotoxins were quantified in cell supernatants. After 1 h exposure, no significant difference was observed between the initial concentrations and the final concentrations of DON and ZEA in both the mono- and co-exposure conditions (Table 3). After 24 h exposure, a reduction in ZEA was observed as concentrations decreased from 20 μM to 0.03 and 0.06 μM in supernatants from cells exposed to ZEA alone and those treated with DON + ZEA, respectively, whereas no difference was observed for DON (Table 3).

4. Discussion

Currently, there is a strong demand for better assessment of health risks related to multiple mycotoxin exposures, as well as to low concentration exposures on short and long time frames using relevant, appropriate models. In addition, in the field of toxicology, certain hypotheses need to be confirmed or discarded, including the endocrine disrupting effect of some mycotoxins (such as ZEA) and their carcinogenic potential (such as TCTs and ZEA) as the International Agency for Research on Cancer (IARC) classified these mycotoxins in group 3 (*i.e.* unclassifiable as to carcinogenicity in Humans) due to the lack of data [22]. Furthermore, the discovery of exposure or effect bioindicators using high-throughput methods (transcriptomics, proteomics, metabolomics) is strongly expected to offer new arguments for epidemiological assessment of the risks incurred by consumers [23]. In this context, using proliferating HepaRG cells, which are recognized as a pertinent model for toxicological studies [24], we investigated the underlying toxicity mechanisms for low doses of DON and ZEA at the proteome level. The effect of the DON + ZEA mixture was also studied in the same conditions since the toxicity of mycotoxins in a mixture cannot always be predicted based upon their individual toxicities [3,25]. More specifically, we characterized the early proteomic changes (after only 1 h exposure) associated with low-dose exposure to DON and/or ZEA using LC-MS/MS analyses, and we compared the obtained candidate mechanism-based proteins to those identified after 24 h exposure using the same doses.

The first aim of this study was to choose, for each fusariotoxin, a dose that would induce a significant cellular response that can be easily observed by proteomics while limiting cell mortality in order to protect proteomic analyses against events that are strictly related to cell death. Achieving cellular death would not provide relevant insights into the specific molecular mechanisms involved in toxic injury. Therefore, in the context of this study, we used 0.2 and 20 μM for DON and ZEA, respectively, for proteomic experiments and verified that no cytotoxicity was induced on HepaRG cells at these concentrations after 24 h exposure (data not shown). It should be noted that these concentrations are higher than the ones estimated in human blood. For example, Maresca [26] estimated DON concentration to be 1.5 nM in human blood. This estimation was based on the provisional maximum tolerable daily intake (PMTDI) of 1 μg of DON/kg of bw/day, and assuming that toxicokinetics data obtained with pigs orally exposed to DON could be extrapolated to humans. Similarly, Shin et al. [27] predicted ZEA concentration in human blood from a physiologically based pharmacokinetic (PBPK) model for ZEA following oral dosing in rats. The authors calculated that an exposure of 0.0312 mg of ZEA/kg of bw/day was necessary to have a steady-state ZEA concentration in human blood of 0.014 ng/ml. This means that, for a PMTDI of 0.2 μg of ZEA/kg of bw/day, the ZEA blood concentration should be about 280 nM. Furthermore, it has been reported that patients with serum levels of 19–100 g/ml ZEA exhibited an increasing incidence of early thelarche [28]. Noteworthy, as stated, these values in human blood were obtained from PMTDI which may be exceeded according to the ingested quantities of contaminated product. The tested concentrations corresponded to the IC_{10} concentrations determined in a previous study after 48 h exposure [11].

Then, after cell exposures of 1 h and 24 h with DON and ZEA alone and in combination, we performed the LC-MS/MS analyses. After only 1 h exposure to mycotoxins, we observed a small but significant change in the proteomes. By comparison, after 24 h exposure to the mycotoxins, about 3 times more proteins were impacted. Furthermore, very few proteins were shared between the individual and combined exposures as well as between the two exposure durations for a same mycotoxin or the mixture, making it difficult to identify potential exposure markers. This issue is also supported by the fact that, among all the mycotoxin-response proteins, very few proteins were at higher abundances than in the control and none appeared to be secreted. However, our results highlighted 2 proteins at significantly lower abundances in all treatments (except after 1 h ZEA incubation): DNA topoisomerase 1 (P11387) and DNA topoisomerase 2-alpha (P11388). Although these enzymes have been already shown to be inhibited by several compounds including the ochratoxin A mycotoxin [29] and some *Alternaria* mycotoxins [30], these results pointed out an important cellular mechanism linked to DON and ZEA toxicity. Thus, they were not considered as specific response proteins to DON or ZEA, and could rather be part of a generic mycotoxin response system or generic immune system response. Our results also showed that DON and ZEA generated very different proteomic profile changes, alone and in combination, depending on the exposure period. Another noticeable point was that DON induced more proteomic changes than ZEA in HepaRG cells after 1 h exposure, whereas the opposite was observed after 24 h exposure. Furthermore, more proteins were differentially abundant due to the mycotoxin mixture than to the toxins alone after 1 h treatment while the contrary was observed after 24 h exposure. These latter findings might suggest a synergistic or additive effect of the mixture on the proteome of HepaRG cells after 1 h exposure, and an antagonistic effect after 24 h exposure. In a previous study, targeting certain liver-

specific functions, we also observed synergism for the DON + ZEA combination at the gene level on HepaRG cells after 1 h exposure using the same concentrations as in the present study [11].

Analysis of the molecular function and subcellular location annotations was in agreement with the literature indicating that DON is a translational inhibitor that binds to eukaryotic ribosomes and thus inhibits DNA, RNA and protein synthesis [31,32] and may cause impairment of membrane function [33]. On the other hand, ZEA has been reported to mainly target mitochondria and/or lysosomes [34,35]. Nevertheless, in our study, no changes in lysosomes were noticed after ZEA exposure on HepaRG cells whereas important changes in the nucleus were observed. When present together, DON and ZEA appeared to affect, the endoplasmic reticulum (ER) after 24 h exposure. A recent review from Rabilloud et Lescuyer [36] reported that the emerging keywords from several analyses of toxicoproteomic responses to natural products such as drugs are “ER stress” and “mitochondrial responses”, which suggests they are core cellular responses to which many different toxin mechanisms converge. As explained in this review, the ER and mitochondrial stress response are commonly activated by toxicants due to i) the ability of the mitochondria to pump various cationic species that become concentrated within the mitochondrial matrix and to ii) the fact that ER is the site of localization of major metabolizing enzymes such as cytochromes P450, which degrade organic toxicants but may release more toxic products. Interestingly, our results showed that the 4F22 cytochrome P450 (Q6NT55) was one of the proteins affected by ZEA and DON + ZEA after 24 h exposure.

Analysis of the biological process annotations using UniProt and Gene Ontology databases (data not shown) revealed that the DON and ZEA mycotoxins did not initiate a massive stress response in HepaRG cells after 1 h exposure when present individually, as seen by the limited number of differentially abundant stress protein (4 proteins for DON and 3 proteins for ZEA). The DON + ZEA combined 1 h exposure induced more proteomic changes in the human hepatocytes than their individual exposures by, for instance, slightly exacerbating the cell response to stress. A total of 9 proteins involved in the cellular stress response were affected by the mycotoxin combination after 1 h exposure. However, after 24 h exposure, the opposite effect was observed: the individual exposures of DON and ZEA seemed to induce a higher cellular stress response than their combined exposure (20 and 36 proteins with a stress response biological function for DON and ZEA respectively and only 12 proteins for the mixture). In addition, both DON and ZEA significantly reduced the abundance of the multidrug resistance protein 1 (P08183) – that is responsible for decreased drug accumulation in multidrug-resistant cells – after 24 h exposure while the mixture did not. Thus, these results indicated a possible mitigation or protection strategy utilized by the hepatocytes when exposed to the mycotoxin cocktail for long periods of time. This aspect needs further study to better understand what type of regulation is implicated by the cells when they are facing a mycotoxin cocktail. However, despite the very low mycotoxin doses applied in the present study, all treatment conditions (with the exception of ZEA after 1 h exposure) seemed to induce important pathways related to programmed cell death by affecting the abundances of proteins involved in MAPK signaling pathway (including, for example, the 26S proteasome non-ATPase regulatory subunit 8 – P48556 – for DON 1 h, integrin alpha-V – P06756 – and ras-related protein Rap-1A – P62834 – for DON, ZEA and DON + ZEA 24 h or mitogen-activated protein kinase 1 – P28482 – and mitogen-activated protein kinase 3 – P27361 – for ZEA 24 h). Recently, two studies highlighted the relationship between protein phosphorylation and DON immunotoxic effects [37,38], suggesting the need for further studies using phosphoproteomic techniques.

Analysis of the main biological processes by the functional annotation tool DAVID for DON after 1 h exposure was in accordance with both the analysis of the molecular function and location annotations and with the literature (indicating that DON inhibits DNA, RNA and protein synthesis [31,32]). In addition, the obtained results suggested an effect of DON on protein (post) translational modifications, which was in accordance with the study of Graziani et al. [39] reporting that DON affects iNOs protein expression in human enterocytes through an increase in its ubiquitinylation and degradation by the proteasome. After 24 h of DON exposure, more biological processes were affected (and were mostly related to cell adhesion and transport), but none were common to those impacted after 1 h suggesting a cascade of cellular events along this 24 h period. These results seemed to be correlated with the analysis of the subcellular location annotations (highlighting that the cell membrane was one of the main target of DON after 24 h). After 24 h exposure, ZEA was the treatment that induced the most proteomic changes in HepaRG cells, mainly targeting proteins involved in the cellular response to stress. For the mycotoxin mixture, by comparison with the individual exposures, the DON + ZEA-impacted biological processes were more numerous after 1 h and less numerous after 24 h, but in both cases, most of them appeared to be different than for DON or ZEA single exposures. In addition, as for the individual exposures, the DON + ZEA affected biological processes were very different between 1 h and 24 h. Using transcriptomic and proteomic approaches targeting some hepatic-specific functions, we previously noticed that the cellular response of HepaRG cells to acute (1 h) and chronic (14 and 28 days) exposures to DON and/or ZEA was very different depending on the incubation period, doses and the considered mycotoxin or mixture [40,41].

Despite this significant diversity in the effects, it is interesting to notice that some biological processes affected by DON single exposure were also impacted by the mixture after both exposure periods while only one of those affected by ZEA individual exposure was also impacted by the combination after 24 h exposure (and 0 after 1 h). Thus, these results suggested that, when DON and ZEA were present together, DON might inhibit some toxic effects of ZEA while impacting new biological pathways, specific to the mixture. The same trends were observed at the metabolome level by Ji et al. [42]. These authors explored the *endo*- and *exo*-metabolomes of murine macrophages (ANA-1 cells) after 24 h exposure to DON and ZEA alone and in combination and reported that DON, when present simultaneously with ZEA, may inhibit certain toxic effects of ZEA (namely the estrogenic effects), and that new pathways appeared to be affected by the mixture compared to the toxins alone (namely palmitic acid metabolism). Nevertheless, their results showed that amino acid metabolism and glycometabolism were the two dominant pathways affected by the three conditions. Here, even if after 24 h exposure, all treatment conditions altered the abundances of some proteins involved in the metabolism of various amino acids (as observed from the provided biological processes for each protein using the UniProt database, data not shown), no amino acid biological pathways were significantly affected by the mycotoxins (as observed with DAVID, Table S2). Overall, in this study, we mainly observed a high impact of the mycotoxins and the mycotoxin mixture on cell cycle, cell proliferation and/or cell development, as well as on DNA metabolic processes. However, for DON alone, which is known to induce inflammation and upregulation of several cytokines in numerous cell models [43–45], no significant inflammatory response was highlighted by the biological process analysis using DAVID. This result might suggest that the pro-inflammatory effect of DON can only be observed at higher doses than the one used in our study (0.2 μM). Noteworthy, the DON + ZEA mixture induced such a response after 24 h exposure (Table S2). In addition, the estrogenic effect of ZEA is commonly described in the literature [34,42] but it is rarely reported for DON. Nonetheless, in the present study, we observed a reduction in estradiol 17-beta-dehydrogenase 2 (P37059) abundance – which is an enzyme capable of catalyzing the interconversion of testosterone and androstenedione, as well as estradiol and estrone, following DON exposure (after 1 h) but not after ZEA exposure. *In vivo*, some authors reported reproduction impairments induced by DON on animals, such as decrease in litter size and increase in postnatal mortality [4–6]. Furthermore, we also observed that DON and the DON + ZEA mixture affected

19 and 15 proteins involved in cancer after 24 h exposure respectively (P value < 0.05). It would be interesting to verify if this effect is maintained at longer incubation times and leads to a cancer phenotype.

Finally, since we observed significant effects of the toxins on HepaRG cells, mainly on the nucleus from 1 h exposure, we wondered if this was due to a fast mycotoxin uptake by the cells. Mycotoxin quantification by LC-MS/QTOF in the cell medium revealed no changes in ZEA or DON concentrations after 1 h exposure at IC10 while proteomic changes were induced in HepaRG cells. Even more interestingly, after 24 h exposure, no changes in DON concentration were observed neither for DON alone nor for the DON + ZEA mixture. These observations raised the question about the activation mechanism of the cellular response. In this context, some hypotheses can be proposed, i) non-quantifiable trace amounts of the tested mycotoxins using the selected method (detection limit 0.01 μM using the [M-Na] + 319.115 m/z ion in ESI + mode) can enter the cell suggesting high cell sensitivity or ii) binding of the mycotoxins to membrane receptors, as already reported by Maresca [26], or iii) a combination of both mechanisms. The important number of proteins regulated by ZEA after 24 h exposure could be explained by the much lower measured concentration of ZEA in the cell supernatants at the end of this incubation period. This result suggests that ZEA would be easily absorbed or metabolized by the hepatocytes, while DON would not be. Noteworthy, while DON has been shown to enter numerous animal/human cell types, Königs et al. showed that DON is metabolized neither by human primary hepatocytes nor HepG2 cell line [46]. As the HepaRG cell line was used in this study, the observed results may be associated with the studied cell type and suggest that proliferating HepaRG cells do not metabolize DON. These results might be partly explained by the higher log P (or log K_{OW}) value of ZEA compared to DON (namely 3.58 for ZEA and − 0.71 for DON), indicating that ZEA is much more lipophilic than DON and thus can more easily enter the cell membrane.

In humans, very little data are available on the toxicokinetics of DON and ZEA. The major characterized metabolite of DON, de-epoxy DON (DOM-1), is usually found in urine and stools of animals exposed to DON [47] and is produced *via* intestinal or rumen microbiota activity rather than by the liver [32]. Pestka et Smolinski [33] reported that, in humans, drug-metabolizing enzymes such as CYP450 enzymes do not detoxify DON into DOM-1. However, in the liver, DON can be conjugated to glucuronides leading to the formation of a non-toxic detoxification product, glucuronide conjugated-DON (DON-GLU) [48]. Only unmetabolized DON as well as DON-GLU were detected in human urine sample analyses [49]. Furthermore, the liver, as the small intestine, were also reported to be one of the main sites of deacetylation of DON-acetylated derivatives present in cereals (*i.e.* 3-acetyl and 15-acetyldeoxynivalenol), resulting in the generation of DON [50]. Concerning ZEA, human intestinal microflora cultured in a continuous flow system were unable to degrade this mycotoxin [28]. *In vitro*, the α-zearalenol metabolite has been described as the most preponderant metabolite followed by β-zearalenol using the human intestinal epithelial cells, Caco-2 [51]. Additional data on ZEA metabolites in the cell medium and in the intracellular fraction could verify if the cells absorbed or metabolized this mycotoxin after 24 h exposure since the abundance of the glutathione S-transferase A3 (Q16772) following 24 h to ZEA exposure was modified (Table S1). Indeed, this enzyme conjugates toxicants to a glutathione molecule to prevent its binding to the target. In a previous study, at very low cytotoxic doses (corresponding to the maximum level permitted by the European regulation in cereals intended for direct human consumption), we observed high cytotoxicity of DON alone and in a mixture with ZEA on HepaRG cells in chronic exposure conditions (*i.e.* cells treated with the mycotoxins every two days over 14 days), while ZEA alone didn't induce cell mortality [41]. This could be explained as stated above by the fact that DON would not be metabolized or neutralized by the proliferating HepaRG cells, contrary to ZEA.

5. Conclusion

In this study, proteomic mechanisms underlying the observed cytotoxic effects of low doses of DON and ZEA in proliferating HepaRG cells were revealed using tandem mass spectrometry. The results showed that, despite the diversity of cellular mechanisms involved in the response to the mycotoxins alone and in combination, some similar proteins and thus biological processes were shared between DON alone and the combined DON + ZEA treatment, while no similarities were observed between ZEA-treated cells and DON + ZEA-treated cells. Moreover, these results also revealed that human hepatic cells seem to be very sensitive to DON and ZEA, as highlighted by the observed proteomic changes after only 1 h exposure to low mycotoxin doses thus confirming that very low doses were able to impair cellular homeostasis. These findings also showed that different cellular pathways responded to the different single and combined mycotoxins and to the different incubation periods, emphasizing the need to further explore the regulation capacities of the cells with more complex incubation kinetics and by combining “omics” tools. This innovative, combined analysis of the toxicity, global proteome changes and mycotoxin quantification has specifically revealed that DON was able to induce toxicity in acute conditions affecting the cell at the proteomic level while it was not apparently absorbed by the proliferating HepaRG cells. In conclusion, this raises the question as to whether a DON-sensing mechanism exists and should be further investigated.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

The http://dx.doi.org/10.1016/j.jprot.2017.11.025 associated with this article can be found, in online version.

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Appendix A. Supplementary data

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