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ABSTRACT

Aflatoxins are a group of mycotoxins that have major adverse effects on human health. Aflatoxin B1 (AFB1) is the most important aflatoxin and a potent carcinogen once converted into a DNA-reactive form by cytochrome P450 enzymes (CYP450). AFB1 biosynthesis involves the formation of Versicolorin A (VerA) which shares structural similarities with AFB1 and can be found in contaminated commodities, often co-occurring with AFB1. This study investigated and compared the toxicity of VerA and AFB1, alone or in combination, in HepG2 human liver cells. Our results show that both toxins have similar cytotoxic effects and are genotoxic although, unlike AFB1, the main genotoxic mechanism of VerA does not involve the formation of DNA double-strand breaks. Additionally, we show that VerA activates the aryl hydrocarbon receptor (AhR) and significantly induce the expression of the CYP450-1A1 (CYP1A1) while AFB1 did not induce AhR-dependent CYP1A1 activation. Combination of VerA with AFB1 resulted in enhanced genotoxic effects, suggesting that AhR-activation by VerA influences AFB1 genotoxicity by promoting its bioactivation by CYP450s to a highly DNA-reactive metabolite. Our results emphasize the need for expanding the toxicological knowledge regarding mycotoxin biosynthetic precursors to identify those who may pose, directly or indirectly, a threat to human health.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by moulds that may contaminate food commodities. Aflatoxins are a group of mycotoxins synthesized mainly by moulds from *Aspergillus flavus* and *A. parasiticus* species which can contaminate crops such as maize, rice and nuts, typically in tropical and sub-tropical areas (Schrenk et al., 2020). Among the aflatoxins, aflatoxin B1 (AFB1) receives particular attention due to its high carcinogenic potential for humans (IARC, 1976; Ostry et al., 2017). The health issues related to the presence of AFB1 in food commodities are major and include carcinogenicity, impaired development, immunotoxicity, and even death in case of severe acute exposure (Meissonnier et al., 2008; Schrenk et al., 2020). As a consequence, its presence is monitored and regulated in food and feed in Europe and other parts of the world. In Europe for example, safe levels of AFB1 in food vary depending on the matrix and range from 0.1 μg/kg in processed cereal-based foods and baby foods to 8 μg/kg in groundnuts to be subjected to physical treatment, before human consumption or use as an ingredient in foodstuffs (Commission Regulation (EC) No 1881/2006).

The biosynthesis of AFB1 involves multiple enzymatic steps and the accumulation of different precursor molecules. Versicolorin A (VerA) is a key precursor as its accumulation is an important parameter for triggering the final steps of AFB1 synthesis, however, it has been reported that the conversion of VerA to AFB1 is not complete in certain *Aspergillus* species (Conradt et al., 2015; Lee et al., 1976). Therefore, both mycotoxin can co-occur and VerA can even be detected at a higher concentration than AFB1 in certain food commodities (Abdallah et al., 2017; Abia et al., 2013; Janić Hajnal et al., 2020). The data on the toxicity of VerA are limited, but suggest a high toxic potency of this molecule, with the induction of severe genotoxicity (e.g. DNA damages) and cytotoxicity in different human cell-lines such as the Caco2, HCT116, LS-174T intestinal cell-lines, A549 lung cell line and HepG2 liver cell line

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Abbreviations

AhR	aryl hydrocarbon receptor
AFB1	aflatoxin B1
AFBO	exo-AFB1-8, 9-epoxide
CALUX	Chemical Activated LUciferase gene eXpression
CYP1A1	cytochrome P450 1A1
CYP450s	cytochrome P450
DMSO	dimethyl sulfoxide
DSB	double strand break
RT-qPCR	Reverse Transcription – quantitative Polymerase Chain Reaction
ROS	reactive oxygen species
RS	replication stress
UGT1A6	UDP-glucuronosyltransferase 1-6
VerA	versicolorin A

(Gauthier et al., 2020; Jakšić et al., 2012; Theumer et al., 2018). Because both molecules can co-contaminate food commodities, comparative toxicity studies between AFB1 and VerA are needed to determine if the latter may pose a threat to human health. Moreover, it is of interest to assess if combined toxicity can occur. Particularly in organs relevant to AFB1's toxicity such as the liver.

From a chemical-structural point of view, AFB1 is characterized by having a furofuran-ring with a double bond at the 8,9-position (Supplementary Fig. 1). This double bond is converted *in-vivo* into the highly reactive exo-AFB1-8, 9-epoxide (AFBO). The bioactivation of AFB1 to AFBO is mediated by cytochrome p450 enzymes (CYP450s), mainly in the liver. The reactive AFBO is responsible for covalent DNA and protein binding that can lead to DNA mutations and cytotoxicity. Several human epidemiological studies have identified a strong mechanistic link between exposure to AFB1, its genotoxic effects and, a mutation in a specific codon of the p53 tumour-suppressor gene, which abrogates the function of the tumour repressor and contributes to the progression to hepatocellular carcinoma (Jackson et al., 2003; Stern et al., 2001). Alike AFB1, VerA features a double bond in its final furofuran ring (Supplementary data 1), but it is unclear if its genotoxic effects are dependent on its bioactivation by CYP450s (Gauthier et al., 2020).

CYP450s are a large family of enzymes involved in the biotransformation of xenobiotics and endogenous chemicals. The abundance and activity of CYP450s are partially inducible through a receptor-mediated mechanism, involving different nuclear receptors such as pregnane X receptor (PXR), the constitutive androstane receptor (PXR), and the aryl hydrocarbon receptor (AhR). Several studies have suggested that AFB1 activates these nuclear receptors, although this has not been firmly established (Arenas-Huertero et al., 2019; Ayed-Boussema et al., 2012; Mary et al., 2015). Indeed, a recent study revealed that VerA, but not AFB1, can alter the human intestinal cell line Caco-2 transcriptome inducing a significant overexpression of several gene targets of the AhR (Gauthier et al., 2020). Among these genes were CYP1A1, CYP1A2, CYP1B1, UDP-glucuronosyltransferase 1–6 (UGT1A6), interleukin 17F (IL17F) and indoleamine 2,3-dioxygenase (IDO1). The expression of these genes was not affected by the exposure to AFB1. The same study showed that VerA, unlike AFB1, induces cyto- and genotoxic effects independent of p53 activity. These results suggested that the mechanism of toxicity of both toxins is not the same and that the VerA-mediated activation of AhR might be an important part of its toxicity. Besides, shedding a light on a potential role of the AhR in the toxicological mode-of-action of AFB1 and VerA it may also provide a better understanding of their genotoxic effects and in addition may help to further understand other adverse effects.

In the present study, we aimed at investigating the individual cyto- and genotoxic effects of VerA in liver cells, determine if VerA was able to activate AhR as well as understanding the consequences of such activation when cells are exposed to VerA and AFB1 simultaneously. The HepG2 hepatocarcinoma cell-line was chosen as a model for its human and organ relevance, the fact that it expresses CYP450s related to the bioactivation of AFB1 and its wild-type p53 tumour suppressor protein status (Boehme et al., 2010; Westerink et al., 2010; Westerink and Schoonen, 2007).

2. Material and methods

2.1. Chemicals

AFB1 and actinomycin D were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was purchased from Wellington laboratories (Guelph, Canada). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Valkenswaard, The Netherlands). Analytical grade chloroform, acetic acid and acetonitrile were purchased from Fischer Scientific (Illkirch, France) Stock solutions of the toxins were prepared in DMSO and stored at −20 °C until used.

2.2. Synthesis and purification of versicolorin A

VerA was obtained from wheat which was fermented by a pathway-blocked strain of *Aspergillus parasiticus* that specifically accumulates VerA (A. parasiticus SRRC 0164). VerA was extracted from wheat using chloroform and purified using an HPLC system following an in-house protocol described elsewhere (Gauthier et al., 2020; Theumer et al., 2018). Briefly, after pre-culture in plates containing malt extract agar at 28 °C for 7 days, inoculums were transferred and dispersed in autoclaved wheat and incubated at 28 °C for 7 days. Then, VerA was extracted from wheat and mycelium using chloroform, and the extract was filtered, clarified and evaporated to dryness as previously described (Theumer et al., 2018). The VerA purification was performed with an Ultimate 3000 HPLC system (Dionex/ThermoScientific, Courtaboeuf, France) using a C18-2 semi-preparative column (7.8 mm id × 25 cm, 5 μm resin, Interchim, Montluçon, France) equilibrated in 0.1% acetic acid (solvent A) and acetonitrile (solvent B). VerA was eluted using 47% solvent B for 17 min and a gradient of 47–50% solvent B for 14 min at 4.2 mL/min. The column was rinsed with 90% solvent B for 4 min, and the elution gradient returned to its initial value in 10 min and maintained constant until the end of the run (15 min). After identification using an internal standard (pure VerA), the multiple fractions containing VerA were collected with an ultimate 3000 Fraction Collector (Dionex/ThermoScientific, Courtaboeuf, France) and pooled. Then the solvents were evaporated at low pressure with a Rotavapor® R-215 (Büchi, Flawil, Switzerland). Before toxicity experiments, the identity and purity of VerA obtained were verified by several methods described previously (Theumer et al., 2018). Stock solution aliquots of VerA were prepared at 10 mM using DMSO (Sigma, St Quentin Fallavier, France) and kept at −20 °C until used.

2.3. Cell lines and culturing

The HepG2 human liver hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (Manassas, Virginia, United-States). The Dioxin Responsive$_{human}$ Chemical Activated LUciferase gene eXpression (DR$_{human}$ CALUX) cell line consists of HepG2 cells stably transfected with an AhR-responsive reporter and a geneticin resistance plasmid (Budin et al., 2021). The DR CALUX cell line consists of rat hepatoma H4IIE-cells stably transfected with an AhR-responsive reporter and geneticin resistance plasmid (Murk et al., 1996). The U2OS-based p53 CALUX cell-consists of human osteosarcoma U2OS cells stably transfected with a p53-responsive reporter construct and geneticin resistance plasmid line (van der Linden et al., 2014). The HepG2-based p53 CALUX cell-line (this study) was obtained after the

stable transfection of wild-type HepG2 cells with a p53-responsive construct (12xp53RE) described by van der Linden et al. (2014) insulated with sequences of the chicken hypersensitive site-4 gene (Arumugam et al., 2009) and the geneticin resistance plasmid pSG5-neo described elsewhere (Sonneveld et al., 1998).

Wild-type HepG2, DR_{human} CALUX, HepG2-p53 CALUX and U2OS-p53 CALUX cells were maintained in DMEM:F-12 (Gibco) medium supplemented with 7.5% FCS, 10% non-essential amino acids (NEAA), respectively, and streptomycin (10 mg/mL) plus penicillin (10U/mL) antibiotics (referred to as maintenance medium). DR CALUX cells were maintained in α-MEM (Gibco) medium supplemented with 10% FCS and streptomycin (10 μg/mL) plus penicillin (10U/mL) antibiotics. The assay medium used for exposure to toxins in CALUX assay consisted of phenol-free DMEM:F12 (Gibco) medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS), 10% NEAA and streptomycin (10 mg/mL) plus penicillin (10U/mL) antibiotics. Cultures were maintained under standardized conditions in a humidified atmosphere with 5% CO_2 at 37 °C. To reduce background AhR activity in DR and DR_{human} CALUX cells exposed for 4 h, conditioned medium was used as assay medium and consists of sterile-filtered assay medium used to culture DR or DR_{human} CALUX cells (accordingly) for 48 h (Pieterse et al., 2013).

2.4. Cytotoxicity determination

Cell viability was measured using a luminescence-based assay (CellTiter-Glo, Promega, United-States) as already described (Le et al., 2018). The CellTiter-Glo assay is based on the determination of ATP-levels as an indicator of cell viability and metabolic activity. The assay was performed according to the manufacturer's instructions. Briefly, sub-confluent cells seeded in a 96-well plate were exposed to increasing concentrations of toxins (from 0.1 μM to 10 μM in log increments) during 24 h after which reagent were added before luminescence measurement. Concentrations inducing cytotoxicity greater than 20% on average were not considered.

2.5. Gene expression analysis

Sub-confluent wild-type HepG2 cells (70%–95% confluence) seeded in 6-well plates were exposed to 1 μM AFB1, VerA or solvent control (DMSO) in assay medium for 24h. Cells were then washed twice in PBS and total RNA was extracted using Extract-all reagent (Eurobio, Les Ulis, France). RNA isolation and RT-qPCR were performed as described elsewhere (Alassane-Kpembi et al., 2017a; Pierron et al., 2016). Data analysis was carried out using LinRegPCR freeware (Ramakers et al., 2003), and normalized against the reference gene Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1). Primers are presented in Table 1.

2.6. CALUX analyses

The CALUX assay measures the activation of specific pathways, here the AhR and p53 pathways using the DR CALUX and p53 CALUX assays respectively. In the DR and p53 CALUX assays, the amount of luciferase activity measured is directly correlated to the transcriptional activity of the ligand-activated AhR or p53 protein. CALUX reporter cells from continuous culture were resuspended in assay medium to a density of 3×10^5 cells/mL (DR CALUX) or 2×10^5 cells/mL (DR_{human} and HepG2-p53 CALUX) or 1×10^5 cells/mL (U2OS-p53 CALUX). Ninety-six-well plates were seeded with 100 μL of the cell suspension per well and incubated under standard conditions (5% CO_2, 37 °C) for 24h to reach sub-confluence. Then, cells were exposed in triplicates to a 9-step dilution series in log unit increments of each toxin prepared in assay medium or conditioned medium for 4 h exposure in DR and DR_{human} CALUX. On each plate, a dilution series of the reference compound (2,3,7,8-TCDD for DR and DR_{human} CALUX and actinomycin D for HepG2-p53 CALUX and U2OS-p53 CALUX) was included. Vehicle (DMSO) never exceeded 1% in the final dilution. After 4h (DR and DR_{human} CALUX) or 24 h of exposure (DR, DR_{human} and HepG2 and U2OS p53 CALUX assays), cells were lysed using 5 min shaking in a Triton X-100 buffer. The luciferase activity was measured after the addition of a luciferin-containing solution using a luminometer plate reader (Berthold, Bad Wildbad, Germany).

The software GraphPad Prism (San Diego, California USA) was used for dose-response modelling of DR_{human} CALUX receptor-mediated assay. The modelling employs four parameters nonlinear regression model (Y=Bottom of the response + (Top of the response – Bottom of the response)/(1 + 10^((LogEC50-X)*Hill Slope) results are expressed as % percentage of maximal induction of the reference compound 2,3,7,8-TCDD (100% corresponding to full receptor activation). Assessed compounds were considered "positive" in the DR_{human} and DR CALUX assays when the response of at least one concentration was above the determined threshold of 5% 2,3,7,8-TCDD-equivalent activity with a standard deviation (SD) lower than 20%. For HepG2-p53 and U2OS-p53 CALUX assay, the fold-induction per well was calculated by dividing the average Relative Light Units (RLU) level of the tested compound by the average RLU of the solvent control DMSO. In both p53 CALUX assays, Actinomycin-D was used as a positive control and tested compounds were considered "positive" when the response of at least one concentration was above the determined 1.5-fold induction threshold and SD < 20% (van der Linden et al., 2014).

2.7. γH2AX in cell-western

The yH2AX in-cell western assay (yH2AX ICW) is based on the quantification of the phosphorylation of the histone H2AX (γH2AX) which reflects a global genotoxic insult. The γH2AX ICW assay was performed as previously described (Payros et al., 2017). Briefly, 3.2×10^4 wild-type HepG2 cells were seeded per well in 96-well plates using assay medium. After 16 h of incubation under standardized conditions, cells were exposed to toxins for 24 h (in triplicates), fixed with 4% paraformaldehyde (Electron Microscopy Science, Pelanne Instruments, France) in PBS and permeabilized with 0.2% Triton X-100. After blocking (MAXblock Blocking Medium supplemented phosphatase inhibitor PHOSSTOP and 0.1 g. L-1 RNAse A), cells were incubated for 2 h with rabbit monoclonal anti-γH2AX (Clone 20E3, Cell signalling) primary antibody. Detection was carried out using infrared fluorescent dye conjugated to goat anti-rabbit antibody (CF770, Biotium), and RedDot2 was added for DNA quantification (Biotium). After 1h of incubation, fluorescence was measured using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec, Les Ulis, France). γH2AX and RedDot2 signals from treated wells were expressed as fold change compared with negative controls. RedDot2 signal was used as a measure of in-well cell viability and expressed as relative cell count (RCC or final cell count ((treated)/final cell count (control) × 100) assessed by automated fluorescence. γH2AX was expressed in fold-change induction of each concentration of toxin relative to vehicle control, in those exposure conditions where cell viability was higher than 50%. The tested compounds were considered "positive" when the response of at least one

Table 1
Primer sequences for SYBR Green quantitative PCR analysis.

Gene	Accession number	Size (bp)	Sequence
hCYP1A1	NM_000499	102	F – GGTGTTAAGTGAGAAGGTGATTATC R – AGCAGGATAGCCAGGAAGAG
hCYP1A2	NM_000761.5	134	F – GGACTTCTTCCCCATCCTTCG R – GGACACTGTTCTTGTCAAAGTCC
hCYP3A4	ENST00000651514.1	95	F - GGATCCATTCTTTCTCTCAATAA R - AATTTGTAACTTCTCTTGGAAAC
hHPRT1	NM_000194	59	F – AGTAATTGGTGGAGATGATCTCTCAA R – TGACCAAGGAAAGCAAAGTCTG

concentration was above the determined 1.2-fold induction threshold and SD < 20% (Khoury et al., 2013).

2.8. Data handling

In CellTiter-Glo, CALUX and yH2AX assays, any tested concentrations resulting in average values (%cell-viability, %2,3,78-TCDD, fold-induction) above the aforementioned threshold values deemed to be statistically significant from the DMSO control. To compare gene expression levels and the responses between individual toxins and AFB1/Vera combination in CellTiter-Glo, CALUX and yH2AX assays, statistical analyses were performed using GraphPad Prism built-in functions for *t*-test statistical analyses. Differences between groups deemed to be significant with a p-value ≤ 0.05 (*); ≤0.01 (**); ≤0.001 (***).

3. Results and discussion

3.1. Aflatoxin B1 and versicolorin A show similar cytotoxicity in HepG2 cells

The time and concentration-dependent cytotoxic effects of AFB1 and VerA were determined to define non-cytotoxic exposure conditions for subsequent experiments. HepG2 cells were exposed to increasing concentrations of AFB1 or VerA during 24h or 48h and the effect on cell viability was determined using the CellTiter-Glo® assay. Results presented in Fig. 1 show that, for both AFB1 and VerA, the percentage of viable cells at 48 h decreased at concentrations higher than 1 μM in a dose-response manner. After 24 h of incubation, a slight effect was visible but no concentration resulted in a decrease in viability higher than the threshold of 20%. The maximal cytotoxicity after 48h was obtained at 10 μM for both toxins. After 48h of exposure to 30 μM of toxin we also observed an increase in cell-viability. We believe, however, that this is an unspecific effect that occurs at high concentrations of toxins after 48h of incubation that affects the readout of the CellTiter-Glo®. For subsequent experiments, the incubation time was set at 24 h. The cytotoxicity we report for VerA after 48h of incubation is in line with what have been reported by Gauthier et al. (2020) in human intestinal HCT116 and Caco-2 cell lines using the same method. At 24h of incubation, Theumer et al. (2018) reported higher cytotoxicity for VerA in HepG2 cells using a different method (fluorescence-based). Overall, our results show that in HepG2 cells, VerA and AFB1 have similar cytotoxic potency which results in significant cytotoxic effects after 48h of incubation.

3.2. Comparative genotoxicity of versicolorin A and aflatoxin B1

We subsequently compared the genotoxicity of VerA and AFB1 in HepG2 cells through the evaluation of the induction of H2AX phosphorylation, Serine15-p53 phosphorylation and p53 tumour repressor induction, and compared these results with p53 tumour repressor induction in cells devoid from phase I enzymes (U2OS cells).

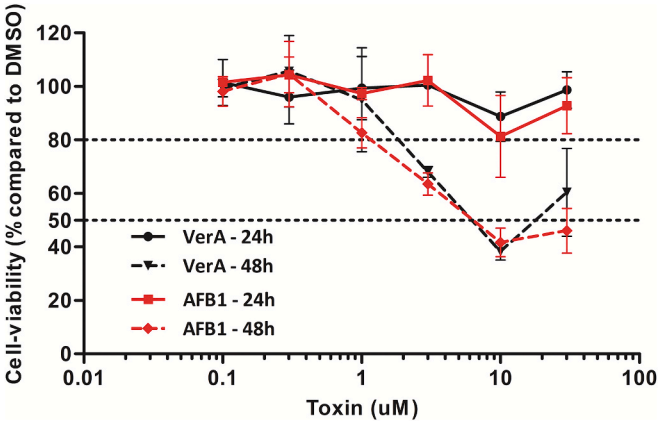

Fig. 1. Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on cell viability in HepG2 cells measured using the CellTiter-Glo® assay (mean ± SD).

We observed that in HepG2 cells both AFB1 and VerA elicited a concentration-dependent increase of yH2AX levels (Fig. 2A) and p53 transcriptional activity (Fig. 2B). HepG2 cells exposed to 1 μM of each toxin for 24h also showed a higher abundance of phosphorylated-p53 (Ser15, Fig. 2C). AFB1 was a more potent inducer of the phosphorylation of H2AX and p53 than VerA, whereas the p53 transcriptional response induced by both toxins was similar. In the case of yH2AX induction, the lowest tested concentration at which levels significantly induced genotoxicity, referred as lowest observed effect concentration (LOEC; first tested concentration >1.2-fold induction in the yH2AX-ICW assay), were factor 33 different, equal to 0.3 μM and 10 μM for AFB1 and VerA respectively.

The LOEC values we found for AFB1 are in the range of those obtained by others in yH2AX-ICW assays performed in HepG2 cells (Khoury et al., 2013; Theumer et al., 2018). In the HepG2-p53 CALUX assay the LOEC (first tested concentration >1.5-fold induction in the HepG2-p53 CALUX assay) was equal to 1 μM for both AFB1 and VerA. In the case of AFB1, our reported LOEC value is in line with those obtained with another HepG2-based p53 reporter gene assays (Westerink et al., 2010) and an ELISA-based assay (Boehme et al., 2010). Regarding induction of p53 Ser15 phosphorylation by AFB1 and VerA, our results are in line with previous reports in intestinal HTC116 cells (Gauthier et al., 2020). Additional experiments using the p53 CALUX U2OS cells, a cell line that does not show appreciable levels of CYP450s activity (van der Linden et al., 2014; van Vugt-Lussenburg et al., 2018), showed that neither AFB1 nor VerA increased p53 transcriptional activity (Supplementary Fig. 2). This indicates that the metabolic status of the exposed cells defines the presence and magnitude of p53 induction for both toxins. Our results suggest that both AFB1 and VerA are genotoxic to human liver cells following cellular events dependent on the metabolic status of the cell. However, the molecular mechanisms leading to these genotoxic effects seem to differ, as AFB1 induced greater phosphorylation of the key proteins H2AX and p53 while both toxins induced similar levels of p53 transcriptional activity.

The genotoxic mechanism of AFB1 is well known. The bioactivation of AFB1 by CYP450s results in the production of AFBO, a metabolite that spontaneously and irreversibly attaches to guanine residues to generate highly mutagenic DNA adducts. These adducts lead to the promotion of a mutational effect at the codon 249 in exon 7 of the p53 tumour suppressor gene. Ultimately, this mutation provokes the development of hepatocellular carcinoma which is a major adverse outcome related to aflatoxin exposure (Hamid et al., 2013). Contrastingly, little is known regarding the molecular mechanisms leading to the genotoxicity of VerA. Previous reports on VerA and AFB1 toxicity suggested a different mode of action for each mycotoxin (Gauthier et al., 2020; Theumer et al., 2018). The study of Gauthier et al. (2020) also revealed a differential transcriptomic response in intestinal Caco-2 cells exposed to these toxins, with VerA being a significantly stronger disruptor of gene expression, as well as being more genotoxic. Furthermore, VerA direct and indirect genotoxic effects were observed in cells with limited bioactivation capacity, suggesting that VerA may be genotoxic as a parent compound independent of its bioactivation by CYP450s (Gauthier et al., 2020; Theumer et al., 2018). Nonetheless, the formation of DNA-reactive species during VerA metabolism by CYP450 cytochromes is structurally possible (reactive double-bond, Supplementary Fig. 1), but has not been confirmed yet.

Besides the characteristic AFB1-like reactive double-bond, other structural features of VerA (anthraquinone with β-hydroxy, Supplementary Fig. 1) suggest that it would be able to induce mitochondrial respiratory chain uncoupling associated with a subsequent induction of severe oxidative stress (Gauthier et al., 2020; Theumer et al., 2018). Therefore, it is likely that reactive oxygen species (ROS) accumulating

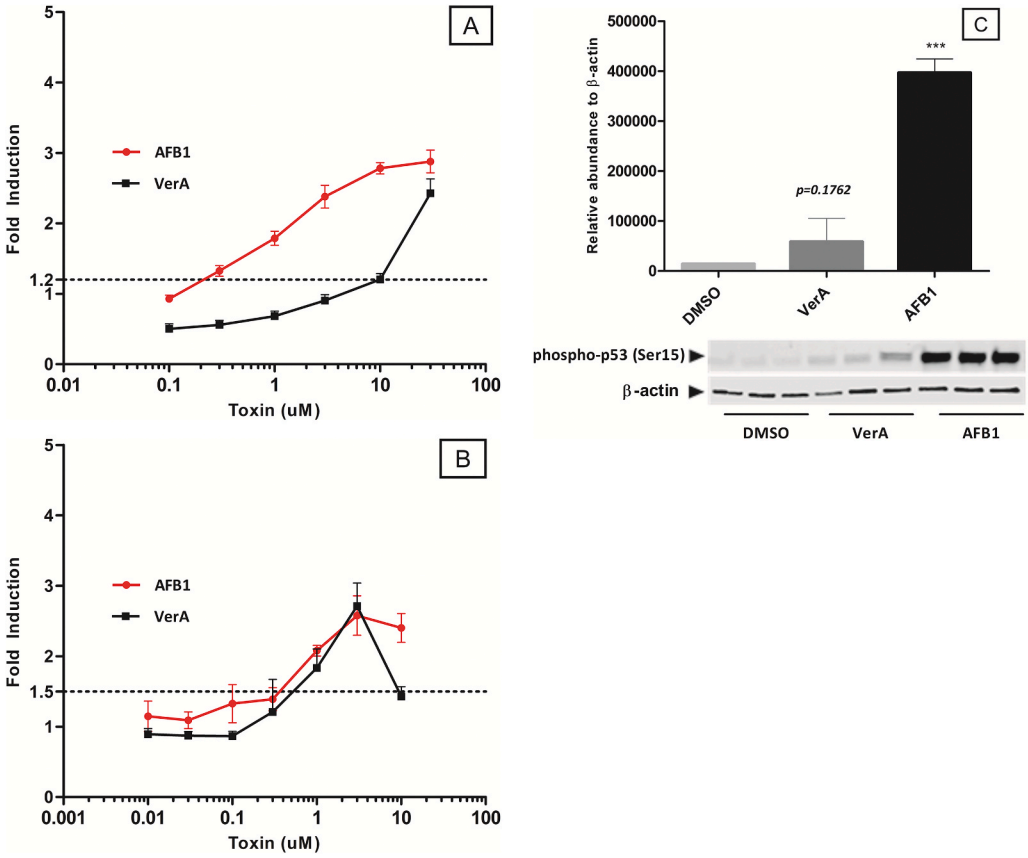

Fig. 2. Individual effects of aflatoxin B1 (AFB1) and versicolorin A (VerA) on (A) H2AX phosphorylation in HepG2 cells measured using the yH2Ax-ICW assay, on (B) p53 transcriptional activity in the HepG2-p53 CALUX assay and on (C) on the p53-Ser15 phosphorylation in wild-type HepG2 cells exposed to 1 μM of each toxin, or to vehicle control (DMSO) for 24h (mean ± SD). ***: p-value ≤ 0.001 (*t*-test).

during this process can induce DNA damage. Besides, the ability of VerA to induce higher oxidative stress than AFB1 was demonstrated in intestinal Caco-2 cells (Gauthier et al., 2020). Two main pathways, namely the ATM-Chk2 axis and the ATR-Chk1 axis govern p53 and H2AX phosphorylation and subsequent DNA damage-mediated cell cycle arrest. Of these, the ATM-Chk2 axis responds to DNA double-strand breaks (DSBs) whereas the ATR-Chk1 axis responds to replication stress (RS; Shaltiel et al., 2015). The p53 protein can be phosphorylated at different sites depending on the state of activation. The phosphorylation of the Ser15 is known to be mediated by Chk2 and is an early event which is specific of DSBs-dependent p53 activation. Also, the level of phosphorylation of p53 at Ser15 is known to correlate with the degree of genotoxic stress induced by DSBs (Ichwan and Ikeda, 2008). Gauthier et al. (2020) has shown that during the genotoxic stress induced by AFB1 and VerA, ATR is activated before ATM, suggesting that DNA DSBs occur after RSs. In their study, VerA was able to induce an earlier and greater RS than AFB1 (8h for VerA vs 16h for AFB1), which in turn might be the trigger to DSB formation. Our results support these findings and suggest a similar mode of action in the liver emphasizing that despite sharing structural features, both toxins show some differences in their genotoxicity mechanisms. While AFB1 genotoxic effects are attributed to a bioactivated metabolite VerA genotoxic effects may be attributed to both a bioactivated metabolite (Supplementary Fig. 1, green dashed circle) and the induction of oxidative stress through its β-hydroxylated anthraquinone structure (Supplementary Fig. 1, blue dashed circle). Therefore, future experiments aiming at better understanding the toxicity of VerA in human liver cells should investigate such aspect. This may be achieved through, example given, the quantification of ROS and the detection of biomarkers indicative of DNA specific oxidative stress such as the oxidized derivative of deoxyguanosine 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).

3.3. Comparative induction of AhR and expression of CYP450 enzymes by versicolorin A and aflatoxin B1

To study the effect of both toxins on AhR activation, AhR-responsive DR_{human} CALUX reporter cells were exposed to non-cytotoxic concentrations of AFB1 and VerA. As showed in Fig. 3, we did not observe a significant concentration-dependent increase of AhR transactivational activity for AFB1 (1.3% of 2,3,7,8-TCDD equivalent activity) after 4 h of incubation. By contrast, VerA significantly induced AhR-transactivation in a concentration-dependent manner up to 52% of 2,3,7,8-TCDD equivalent activity with a calculated EC50 of 0.9 μM and calculated LOEC of 0.2 μM (>5% 2,3,7,8-TCDD equivalent activity in the DR_{human} CALUX assay). When toxins were incubated for 24 h, a strong decrease in maximal AhR-mediated luciferase induction by VerA was observed (Supplementary Fig. 3A), thus supporting our earlier finding that cellular metabolism negatively influences the concentration of VerA in the cell. The effects of AFB1 and VerA on AhR transactivation were confirmed in another (metabolically competent) AhR-responsive cell-line, the DR CALUX, in which we obtained similar results for both toxins (Supplementary Fig. 3B).

The AhR is a latent cytoplasmic transcription factor involved in xenobiotic sensing, induction of CYP1A1/2 as well as other cellular processes such as cancer progression and control of the cell-cycle (Dietrich and Kaina, 2010; Esser and Rannug, 2015; Larigot et al., 2018). A previous transcriptomic analysis on intestinal Caco-2 cells revealed significant up-regulation of AhR target genes in cells exposed to VerA, including several CYP450 enzymes, whereas AFB1 did not induce such changes (Gauthier et al., 2020). These results suggested an involvement

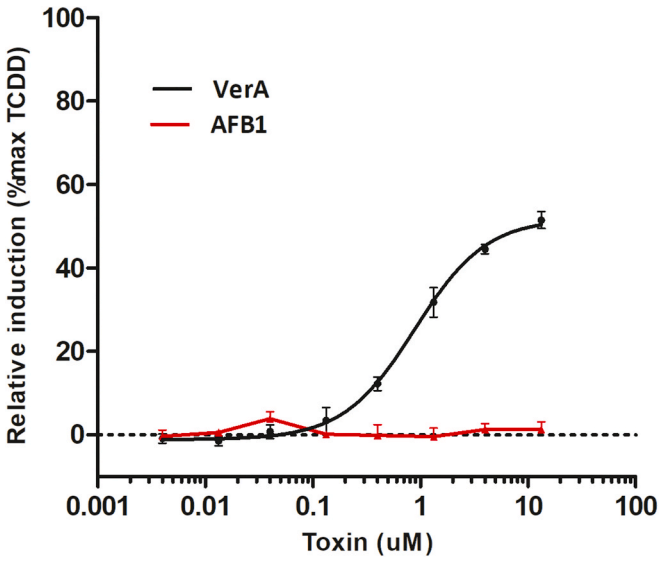

Fig. 3. Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on the AhR-mediated luciferase induction in HepG2 DR_{human} CALUX reporter cells after 4 h of incubation (mean ± SD).

of AhR signalling in VerA toxicity, which has not been investigated yet. Moreover, typical ligands of the AhR, such as dioxins and certain dietary compounds, display a planar chemical structure (Denison and Nagy, 2003), which further suggested that, VerA could interact with the receptor and affect normal cell physiology through activation and/or disruption of normal AhR signalling. The present results indicate that VerA can activate the Ah-receptor which is consistent with gene expression results found in Caco-2 cells (Gauthier et al., 2020).

Then, we determined if VerA-dependent activation of the AhR was associated with the induction of CYP450 enzymes expressed in HepG2. That is CYP1A1/2 and CYP3A4 (Boehme et al., 2010; Westerink and Schoonen, 2007). The results, presented in Fig. 4, indicate a pattern of induction where VerA significantly increased the expression of CYP1A1 (2,4-fold) mRNA whereas AFB1 significantly increased the expression of CYP1A1 (2,2-fold) and CYP3A4 (1,5-fold). In the case of AFB1, results are in line with reported induction of CYP3A4 and CYP1A1 transcription

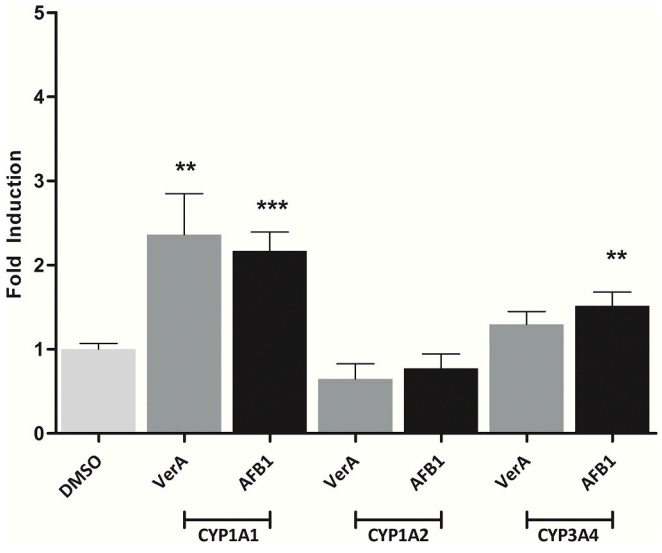

Fig. 4. Effects of 1 μM versicolorin A (VerA) and 1 μM of aflatoxin B1 (AFB1) on the mRNA transcription induction of CYP1A1/2 and CYP3A4 in HepG2 cells (mean ± SD). *: p-value (compared to DMSO control) ≤ 0.05; **: ≤0.01; ***: ≤0.001 (*t*-test).

by AFB1 in different cell types (Ayed-Boussema et al., 2012; Boehme et al., 2010). There was no significant induction of CYP1A2 for both toxins. For VerA, our results in a human liver cell-line are in accordance with those reported by Gauthier and co-workers in human Caco-2 intestinal cells although induction of CYP1A1 by VerA in HepG2 cells was lower (Gauthier et al., 2020). Our results show that VerA can induce CYP1A1 mRNA transcription, opening the question that the toxin may be a possible substrate of this CYP450 isoform, as suggested by others (Gauthier et al., 2020) and our earlier results regarding genotoxicity. In our study, the elevation of CYP1A1 mRNA level was also associated with significantly increased AhR transactivational activity. This suggests an AhR-mediated mechanism for CYP1A1 induction by VerA and/or an effect on AhR signalling and a possible role of the AhR in the genotoxicity of VerA.

The VerA-mediated induction of the AhR also suggests a potential enhancing effect of AFB1 genotoxicity as the ligand-binding activation of the AhR results in increased expression of CYP450s that can catalyse the bioactivation of procarcinogens. This mechanism has been well described in the case of polyaromatic hydrocarbons, such as benzo[a]pyrene, whose AhR-mediated bioactivation by CYP1A1 results in the production of DNA-reactive PAH metabolite forming mutagenic DNA adduct and DSBs (Smit et al., 2017). Therefore, when AFB1 and VerA are present simultaneously, the AhR-mediated CYP1A1 inducing properties of VerA may support the formation of AFB1 reactive metabolites and enhance related DNA damages.

Furthermore, the interaction of VerA with AhR signalling indicates a possible role of the AhR in the AFB1-independent genotoxicity mechanisms of VerA itself. Regarding the hypothesis that certain aspects of VerA genotoxicity may be attributed to DNA damages resulting from oxidative stress, it should be noted that the AhR plays a significant role in the upstream events of p53-mediated cell-cycle progression/apoptosis. Typically through the control and regulation of ROS levels via the induction and repression of certain CYP450s and non-CYP450 enzymes such as UGT1A6 (Nebert et al., 2000). Thus, the alteration of normal AhR signalling by VerA may result in a perturbation of that balance and lead to ROS formation and subsequent elevated oxidative stress. Considering the importance of the AhR in normal and disturbed physiology, in several organs such as the liver or the intestine (Larigot et al., 2018; Safe et al, 2013, 2020) future investigations should aim at further elucidating the relationship of VerA with AhR events related to cell-cycle which may help to better understand the toxicity mechanisms of VerA.

3.4. *Versicolorin A enhances the genotoxicity of aflatoxin B1*

As VerA and AFB1 may co-occur in food, we next studied the effect on cytotoxicity and genotoxicity of VerA in combination with AFB1, using increasing concentrations of AFB1 with 1 μM of VerA. This concentration of VerA induces a significant activation of AhR and CYP1A1 expression over a 24h time-period of exposure but did not have a significant cytotoxic effect nor induced a significant increase of γH2AX or phospho-p53. Results showed that exposure to this mixture resulted in a stronger cytotoxic effect compared to that of 1 μM VerA alone, as well as the effect of AFB1 alone, in particular after 48 h of exposure (Fig. 5). Exposure of HepG2 cells to the mixture resulted in significantly higher levels of γH2AX induction than the effect of 1 μM of VerA alone and the effect of AFB1 alone from 0,1 μM–3 μM (Fig. 6A). The same effect was observed in the HepG2-p53 CALUX where the combination resulted in significantly greater levels of p53 transcriptional activity than 1 μM of VerA alone and 0,1 μM–1 μM of AFB1 alone (Fig. 6B), as well as regarding Ser15-p53 phosphorylation at 1 μM (Fig. 6C). Interestingly, genotoxicity was enhanced especially in those mixtures containing relatively low AFB1 concentrations (0.1 μM–1 μM).

The finding that low concentrations of AFB1 in combination with 1 μM of VerA elicit significant cyto- and genotoxic effects is highly relevant to food hazard assessment. Other studies have highlighted the

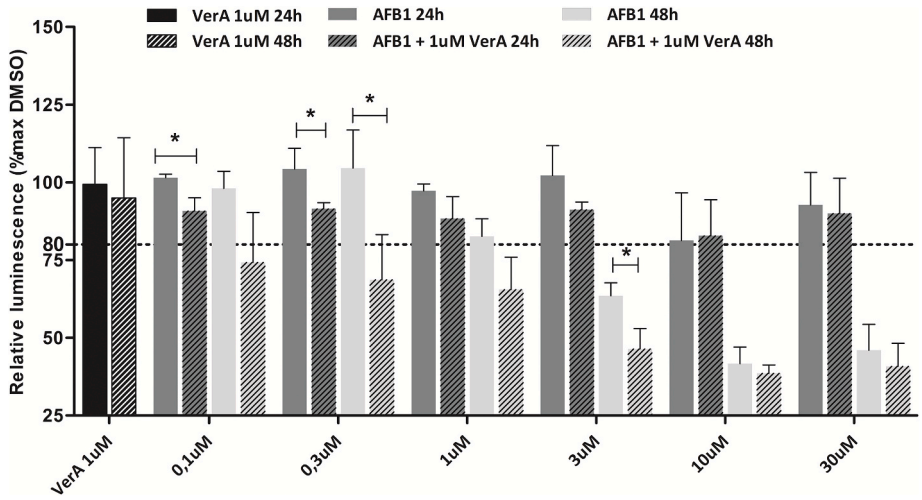

Fig. 5. Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) and mixtures of AFB1 + 1 μM of VerA on cell viability in HepG2 cells measured using the CellTiter-Glo® assay (mean ± SD). *: p-value ≤ 0,05 (*t*-test).

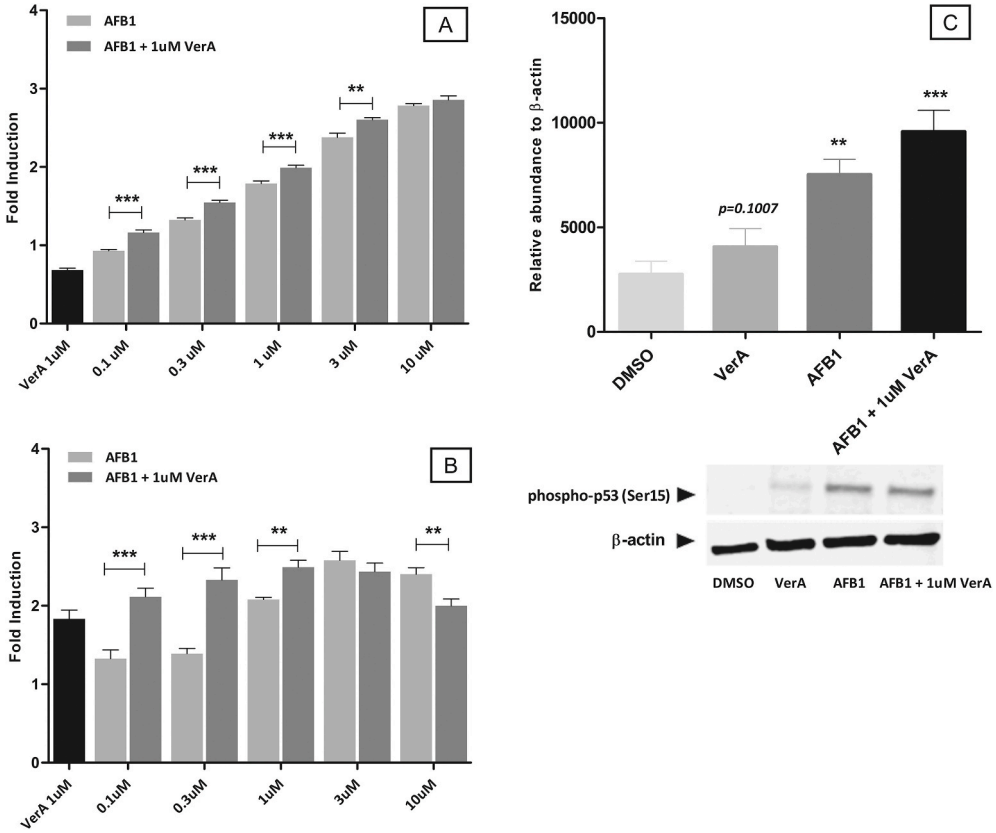

Fig. 6. Combined effects of aflatoxin B1 (AFB1) and 1 μM of versicolorin A (VerA) on (A) H2AX phosphorylation in HepG2 cells measured using the yH2Ax-ICW assay and (B) p53 transcriptional activity in HepG2-p53 CALUX assay and (C) p53-Ser15 phosphorylation in wild-type HepG2 cells compared to their individual effects (mean ± SD). *: p-value ≤ 0.05; **: ≤0.01; ***: ≤0.001 (*t*-test).

effects elicited by the combination of AFB1 with other regulated mycotoxins such as fumonisin B1, ochratoxin B1 and deoxynivalenol (Alassane-Kpembi et al., 2017b; Corcuera et al., 2011; Mary et al., 2015; McKean et al., 2006). Combined effects of AFB1 with fumonisin B1, deoxynivalenol and T-2 toxin on endpoints such as cell viability, cytotoxicity indicated additivity and synergism, although the magnitude may vary depending on the cell system. Combination of AFB1 and carcinogenic mycotoxins, ochratoxin A and fumonisin B1, also suggest enhanced genotoxicity via mechanisms that remain to be understood. Our results are in line with these, but also emphasize that interactions can also occur between toxins from the same family, and that more frequently than not, toxicity from mixtures cannot be explained by the toxicity of individual toxins. This is relevant for regulatory issues since regulations regarding mycotoxins levels in food and crops generally focus on the identification and quantification of a handful of mycotoxins, which are often final fungal metabolites. However, the occurrence and concentrations of numerous biosynthetic precursors of mycotoxins that are normally overlooked are recently being

communicated, revealing their importance (Abdallah et al., 2017; Janić Hajnal et al., 2020; Kovalsky et al., 2016). Mycotoxins are always found as complex mixtures rather than as single compounds in foodstuffs, and there is a need in expanding the toxicological knowledge regarding the toxicity of biosynthetic precursors, and the toxic effect of complex mycotoxin mixtures (Streit et al., 2013). AFB1 and VerA show a similar structure, and it was anticipated that the toxicity profile and mode of action may be similar and thus elicit combined effects (Speijers and Speijers, 2004). However, here we described that VerA and AFB1 do not share the same mechanism of genotoxicity nor the same kinetics in the activation of DNA damage responses. Indeed, our results indicate that VerA increases AFB1 toxicity by promoting its bioactivation and promoting additional genotoxic stress. These new aspects of the toxicity of VerA, different from those of AFB1, could explain that the combined exposure to AFB1 and VerA lead to more deleterious effects than AFB1 alone. Particularly through the increase of p53 transcriptional activity and Ser-15 phophorylation levels (Fig. 6B and C) which may translate to an increased genotoxic stress leading to adverse cellular events such as apoptosis. Future research should be conducted to fully characterise the interaction of VerA and AFB1 and the nature of their interaction at all concentrations. Moreover, it will be important to verify if the AhR activation promoted by VerA is associated with a greater bioactivation of AFB1 or if the interaction relies in their apparent different genotoxicity mechanisms.

4. Conclusion

The toxicological knowledge regarding the effects of AFB1's precursors is scarce at best and, to our knowledge, there have not been studies evaluating the combined genotoxic effect of AFB1 and its precursors in human cells. In this study, we applied *in vitro* bioassays and methods to evaluate and compare the toxic effects of VerA and AFB1. The cytotoxicity and genotoxicity of VerA in HepG2 cells were reflected by the decrease of cell viability and the induction of H2AX phosphorylation, serine15-p53 phosphorylation and p53 tumour repressor transcriptional activity. Differential genotoxic responses suggested differences in mechanisms of action of VerA and AFB1. Unlike AFB1, the main genotoxic mechanism of VerA does not seem to involve the formation of DSBs by VerA-epoxide DNA reactive metabolites. VerA was able to induce the transcription of CYP1A1 via an AhR-mediated mechanism which suggested that VerA can influence AFB1 toxicity by promoting its bioactivation by CYP450s. The addition of 1 μM of VerA to increasing concentrations of AFB1 resulted in significantly enhanced AFB1 cyto- and genotoxic effects thus, supporting the hypothesis that the AhR activation promoted by VerA could be associated with a greater bioactivation of AFB1. The present results pinpoint to a need of expanding the toxicological knowledge regarding mycotoxin biosynthetic precursors with the aim of identifying those who may pose a threat to food safety. Since the exposure to multiple mycotoxins is a rule rather than an exception, our results additionally contribute valuable information to hazard identification and risk assessment in food safety. Besides, the *in vitro* human cell-based bioassays as applied in this study (p53 CALUX and ICW-γH2AX) appears to be relevant methods to efficiently study the genotoxic effects of mixtures of mycotoxins.

CRediT authorship contribution statement

Clémence Budin: Conceptualization, Investigation, Writing – original draft, Formal analysis. **Hai-Yen Man:** Data curation, collection. **Carine Al-Ayoubi:** Investigation, Writing – review & editing. **Sylvie Puel:** Investigation, Writing – review & editing. **Barbara M.A. van Vugt-Lussenburg:** Validation, Writing – review & editing. **Abraham Brouwer:** Writing – review & editing, Supervision, Funding acquisition. **Isabelle P. Oswald:** Writing – review & editing, Supervision, Funding acquisition. **Bart van der Burg:** Writing – review & editing, Supervision, Funding acquisition. **Laura Soler:** Conceptualization, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112258.

References

Abdallah, M.F., Girgin, G., Baydar, T., Krska, R., Sulyok, M., 2017. Occurrence of multiple mycotoxins and other fungal metabolites in animal feed and maize samples from Egypt using LC-MS/MS. J. Sci. Food Agric. 97, 4419–4428. https://doi.org/10.1002/jsfa.8293.

Abia, W.A., Warth, B., Sulyok, M., Krska, R., Tchana, A.N., Njobeh, P.B., Dutton, M.F., Moundipa, P.F., 2013. Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). Food Contr. 31, 438–453. https://doi.org/10.1016/j.foodcont.2012.10.006.

Alassane-Kpembi, I., Puel, O., Pinton, P., Cossalter, A.-M., Chou, T.-C., Oswald, I.P., 2017a. Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. Arch. Toxicol. 91, 2677–2687. https://doi.org/10.1007/s00204-016-1902-9.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O., Oswald, I.P., 2017b. Mycotoxins co-contamination: methodological aspects and biological relevance of combined toxicity studies. Crit. Rev. Food Sci. Nutr. 57, 3489–3507. https://doi.org/10.1080/10408398.2016.1140632.

Arenas-Huertero, F., Zaragoza-Ojeda, M., Sánchez-Alarcón, J., Milić, M., Šegvić Klarić, M., Montiel-González, J.M., Valencia-Quintana, R., 2019. Involvement of Ahr pathway in toxicity of aflatoxins and other mycotoxins. Front. Microbiol. 10 https://doi.org/10.3389/fmicb.2019.02347.

Arumugam, P.I., Urbinati, F., Velu, C.S., Higashimoto, T., Grimes, H.L., Malik, P., 2009. The 3′ region of the chicken hypersensitive site-4 insulator has properties similar to its core and is required for full insulator activity. PLoS One 4. https://doi.org/10.1371/journal.pone.0006995.

Ayed-Boussema, I., Pascussi, J.-M., Maurel, P., Bacha, H., Hassen, W., 2012. Effect of aflatoxin B1 on nuclear receptors PXR, CAR, and AhR and their target cytochromes P450 mRNA expression in primary cultures of human hepatocytes. Int. J. Toxicol. 31, 86–93. https://doi.org/10.1177/1091581811422453.

Boehme, K., Dietz, Y., Hewitt, P., Mueller, S.O., 2010. Activation of P53 in HepG2 cells as surrogate to detect mutagens and promutagens in vitro. Toxicol. Lett. 198, 272–281. https://doi.org/10.1016/j.toxlet.2010.07.007.

Budin, C., Besselink, H., van Vugt-Lussenburg, B.M.A., Man, H.-Y., van der Burg, B., Brouwer, A., 2021. Induction of AhR transactivation by PBDD/Fs and PCDD/Fs using a novel human-relevant, high-throughput DRhuman CALUX reporter gene assay. Chemosphere 263, 128086. https://doi.org/10.1016/j.chemosphere.2020.128086.

Commission Regulation (EC) No 1881/2006, 2006. Setting Maximum Levels for Certain Contaminants in Foodstuffs (Text with EEA Relevance).

Conradt, D., Schätzle, M.A., Haas, J., Townsend, C.A., Müller, M., 2015. New insights into the conversion of versicolorin A in the biosynthesis of aflatoxin B1. J. Am. Chem. Soc. 137, 10867–10869. https://doi.org/10.1021/jacs.5b06770.

Corcuera, L.A., Arbillaga, L., Vettorazzi, A., Azqueta, A., López de Cerain, A., 2011. Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. Food Chem. Toxicol. 49, 2883–2889. https://doi.org/10.1016/j.fct.2011.07.029.

Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309–334. https://doi.org/10.1146/annurev.pharmtox.43.100901.135828.

Dietrich, C., Kaina, B., 2010. The aryl hydrocarbon receptor (AhR) in the regulation of cell–cell contact and tumor growth. Carcinogenesis 31, 1319–1328. https://doi.org/10.1093/carcin/bgq028.

Esser, C., Rannug, A., 2015. The aryl hydrocarbon receptor in barrier organ physiology, immunology, and toxicology. Pharmacol. Rev. 67, 259–279. https://doi.org/10.1124/pr.114.009001.

Gauthier, T., Duarte-Hospital, C., Vignard, J., Boutet-Robinet, E., Sulyok, M., Snini, S.P., Alassane-Kpembi, I., Lippi, Y., Puel, S., Oswald, I.P., Puel, O., 2020. Versicolorin A, a

precursor in aflatoxins biosynthesis, is a food contaminant toxic for human intestinal cells. Environ. Int. 137, 105568. https://doi.org/10.1016/j.envint.2020.105568.
Hamid, A.S., Tesfamariam, I.G., Zhang, Y., Zhang, Z.G., 2013. Aflatoxin B1-induced hepatocellular carcinoma in developing countries: geographical distribution, mechanism of action and prevention (Review). Oncol. Lett. 5, 1087–1092. https://doi.org/10.3892/ol.2013.1169.
IARC, 1976. IARC monographs on the evaluation of the carcinogenic risk of chemicals to man: some naturally occurring substances. IARC Monogr. Eval. Carcinog. Risk Chem. Man 10, 1–342.
Ichwan, S.J.A., Ikeda, M.-A., 2008. Defect in Ser46 phosphorylation of p53 protein: a resistance mechanism against p53 gene transfer in oral Squamous cell carcinoma cells. J. Oral Biosci. 50, 98–106. https://doi.org/10.2330/joralbiosci.50.98.
Jackson, P.E., Kuang, S.-Y., Wang, J.-B., Strickland, P.T., Muñoz, A., Kensler, T.W., Qian, G.-S., Groopman, J.D., 2003. Prospective detection of codon 249 mutations in plasma of hepatocellular carcinoma patients. Carcinogenesis 24, 1657–1663. https://doi.org/10.1093/carcin/bgg101.
Jakšić, D., Puel, O., Canlet, C., Kopjar, N., Kosalec, I., Klarić, M.Š., 2012. Cytotoxicity and genotoxicity of versicolorins and 5-methoxysterigmatocystin in A549 cells. Arch. Toxicol. 86, 1583–1591. https://doi.org/10.1007/s00204-012-0871-x.
Janić Hajnal, E., Kos, J., Malachová, A., Steiner, D., Stranska, M., Krska, R., Sulyok, M., 2020. Mycotoxins in maize harvested in Serbia in the period 2012–2015. Part 2: non-regulated mycotoxins and other fungal metabolites. Food Chem. 317, 126409. https://doi.org/10.1016/j.foodchem.2020.126409.
Khoury, L., Zalko, D., Audebert, M., 2013. Validation of high-throughput genotoxicity assay screening using γH2AX in-cell western assay on HepG2 cells. Environ. Mol. Mutagen. 54, 737–746. https://doi.org/10.1002/em.21817.
Kovalsky, P., Kos, G., Nährer, K., Schwab, C., Jenkins, T., Schatzmayr, G., Sulyok, M., Krska, R., 2016. Co-occurrence of regulated, masked and emerging mycotoxins and secondary metabolites in finished feed and maize—an extensive survey. Toxins 8, 363. https://doi.org/10.3390/toxins8120363.
Larigot, L., Juricek, L., Dairou, J., Coumoul, X., 2018. AhR signaling pathways and regulatory functions. Biochim. Open 7, 1–9. https://doi.org/10.1016/j.biopen.2018.05.001.
Le, T.-H., Alassane-Kpembi, I., Oswald, I.P., Pinton, P., 2018. Analysis of the interactions between environmental and food contaminants, cadmium and deoxynivalenol, in different target organs. Sci. Total Environ. 622–623, 841–848. https://doi.org/10.1016/j.scitotenv.2017.12.014.
Lee, L.S., Bennett, J.W., Cucullu, A.F., Ory, R.L., 1976. Biosynthesis of aflatoxin B1. Conversion of versicolorin A to aflatoxin B1 by Aspergillus parasiticus. J. Agric. Food Chem. 24, 1167–1170. https://doi.org/10.1021/jf60208a017.
Mary, V.S., Valdehita, A., Navas, J.M., Rubinstein, H.R., Fernández-Cruz, M.L., 2015. Effects of aflatoxin B1, fumonisin B1 and their mixture on the aryl hydrocarbon receptor and cytochrome P450 1A induction. Food Chem. Toxicol. 75, 104–111. https://doi.org/10.1016/j.fct.2014.10.030.
McKean, C., Tang, L., Tang, M., Billam, M., Wang, Z., Theodorakis, C.W., Kendall, R.J., Wang, J.-S., 2006. Comparative acute and combinative toxicity of aflatoxin B1 and fumonisin B1 in animals and human cells. Food Chem. Toxicol. 44, 868–876. https://doi.org/10.1016/j.fct.2005.11.011.
Meissonnier, G.M., Pinton, P., Laffitte, J., Cossalter, A.-M., Gong, Y.Y., Wild, C.P., Bertin, G., Galtier, P., Oswald, I.P., 2008. Immunotoxicity of aflatoxin B1: impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicol. Appl. Pharmacol. 231, 142–149. https://doi.org/10.1016/j.taap.2008.04.004.
Murk, A.J., Legler, J., Denison, M.S., Giesy, J.P., Van De Guchte, C., Brouwer, A., 1996. Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. Toxicol. Sci. 33, 149–160. https://doi.org/10.1093/toxsci/33.1.149.
Nebert, D.W., Roe, A.L., Dieter, M.Z., Solis, W.A., Yang, Y., Dalton, T.P., 2000. Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem. Pharmacol. 59, 65–85. https://doi.org/10.1016/S0006-2952(99)00310-X.
Ostry, V., Malir, F., Toman, J., Grosse, Y., 2017. Mycotoxins as human carcinogens—the IARC Monographs classification. Mycotoxin Res. 33, 65–73. https://doi.org/10.1007/s12550-016-0265-7.
Payros, D., Dobrindt, U., Martin, P., Secher, T., Bracarense, A.P.F.L., Boury, M., Laffitte, J., Pinton, P., Oswald, E., Oswald, I.P., 2017. The food contaminant deoxynivalenol exacerbates the genotoxicity of gut microbiota. mBio 8. https://doi.org/10.1128/mBio.00007-17.
Pierron, A., Mimoun, S., Murate, L.S., Loiseau, N., Lippi, Y., Bracarense, A.-P.F.L., Schatzmayr, G., He, J.W., Zhou, T., Moll, W.-D., Oswald, I.P., 2016. Microbial biotransformation of DON: molecular basis for reduced toxicity. Sci. Rep. 6, 29105. https://doi.org/10.1038/srep29105.
Pieterse, B., Felzel, E., Winter, R., van der Burg, B., Brouwer, A., 2013. PAH-CALUX, an optimized bioassay for AhR-mediated hazard identification of polycyclic aromatic hydrocarbons (PAHs) as individual compounds and in complex mixtures. Environ. Sci. Technol. 47, 11651–11659. https://doi.org/10.1021/es403810w.
Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. https://doi.org/10.1016/S0304-3940(02)01423-4.
Safe, S., Jayaraman, A., Chapkin, R.S., 2020. Ah receptor ligands and their impacts on gut resilience: structure–activity effects. Crit. Rev. Toxicol. 50, 463–473. https://doi.org/10.1080/10408444.2020.1773759.
Safe, S., Lee, S.-O., Jin, U.-H., 2013. Role of the aryl hydrocarbon receptor in carcinogenesis and potential as a drug target. Toxicol. Sci. 135, 1–16. https://doi.org/10.1093/toxsci/kft128.
Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., Mazo, J. del, Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Ron,), Leblanc, J.-C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Marko, D., Oswald, I.P., Piersma, A., Routledge, M., Schlatter, J., Baert, K., Gergelova, P., Wallace, H., 2020. Risk assessment of aflatoxins in food. EFSA J. 18, e06040 https://doi.org/10.2903/j.efsa.2020.6040.
Shaltiel, I.A., Krenning, L., Bruinsma, W., Medema, R.H., 2015. The same, only different – DNA damage checkpoints and their reversal throughout the cell cycle. J. Cell Sci. 128, 607–620. https://doi.org/10.1242/jcs.163766.
Smit, E., Souza, T., Jennen, D.G.J., Kleinjans, J.C.S., van den Beucken, T., 2017. Identification of essential transcription factors for adequate DNA damage response after benzo(a)pyrene and aflatoxin B1 exposure by combining transcriptomics with functional genomics. Toxicology 390, 74–82. https://doi.org/10.1016/j.tox.2017.09.002.
Sonneveld, E., van den Brink, C., van der Leede, B., Schulkes, R., Petkovich, M., van der Burg, B., van der Saag, P., 1998. Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. Cell Growth Differ. 9, 629–637.
Speijers, G.J.A., Speijers, M.H.M., 2004. Combined toxic effects of mycotoxins. Toxicol. Lett. 153, 91–98. https://doi.org/10.1016/j.toxlet.2004.04.046.
Stern, M.C., Umbach, D.M., Yu, M.C., London, S.J., Zhang, Z.-Q., Taylor, J.A., 2001. Hepatitis B, aflatoxin B1, and p53 codon 249 mutation in hepatocellular carcinomas from Guangxi, People's Republic of China, and a meta-analysis of existing studies. Cancer Epidemiol. Biomark. Prev. 10, 617–625.
Streit, E., Schwab, C., Sulyok, M., Naehrer, K., Krska, R., Schatzmayr, G., 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. Toxins 5, 504–523. https://doi.org/10.3390/toxins5030504.
Theumer, M.G., Henneb, Y., Khoury, L., Snini, S.P., Tadrist, S., Canlet, C., Puel, O., Oswald, I.P., Audebert, M., 2018. Genotoxicity of aflatoxins and their precursors in human cells. Toxicol. Lett. 287, 100–107. https://doi.org/10.1016/j.toxlet.2018.02.007.
van der Linden, S.C., von Bergh, A.R.M., van Vught-Lussenburg, B.M.A., Jonker, L.R.A., Teunis, M., Krul, C.A.M., van der Burg, B., 2014. Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress. Mutat. Res. Genet. Toxicol. Environ. Mutagen 760, 23–32. https://doi.org/10.1016/j.mrgentox.2013.09.009.
van Vugt-Lussenburg, B.M.A., van der Lee, R.B., Man, H.-Y., Middelhof, I., Brouwer, A., Besselink, H., van der Burg, B., 2018. Incorporation of metabolic enzymes to improve predictivity of reporter gene assay results for estrogenic and anti-androgenic activity. Reprod. Toxicol. 75, 40–48. https://doi.org/10.1016/j.reprotox.2017.11.005.
Westerink, W.M.A., Schoonen, W.G.E.J., 2007. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. Toxicol. Vitro 21, 1581–1591. https://doi.org/10.1016/j.tiv.2007.05.014.
Westerink, W.M.A., Stevenson, J.C.R., Horbach, G.J., Schoonen, W.G.E.J., 2010. The development of RAD51C, Cystatin A, p53 and Nrf2 luciferase-reporter assays in metabolically competent HepG2 cells for the assessment of mechanism-based genotoxicity and of oxidative stress in the early research phase of drug development. Mutat. Res. Genet. Toxicol. Environ. Mutagen 696, 21–40. https://doi.org/10.1016/j.mrgentox.2009.12.007.