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Abstract

Aflatoxin B1 (AFB1) is the most potent natural carcinogen among mycotoxins. Versicolorin A (VerA) is a precursor of AFB1 biosynthesis and is structurally related to the latter. Although VerA has already been identified as a genotoxin, data on the toxicity of VerA are still scarce, especially at low concentrations. The SOS/umu and miniaturized version of the Ames test in *Salmonella* Typhimurium strains used in the present study shows that VerA induces point mutations. This effect, like AFB1, depends primarily on metabolic activation of VerA. VerA also induced chromosomal damage in metabolically competent intestinal cells (IPEC-1) detected by the micronucleus assay. Furthermore, results from the standard and enzyme-modified comet assay confirmed the VerA-mediated DNA damage, and we observed that DNA repair pathways were activated upon exposure to VerA, as indicated by the phosphorylation and/or relocation of relevant DNA-repair biomarkers (γH2AX and 53BP1/FANCD2, respectively). In conclusion, VerA induces DNA damage without affecting cell viability at concentrations as low as 0.03 μM, highlighting the danger associated with VerA exposure and calling for more research on the carcinogenicity of this emerging food contaminant.

Keywords: Mycotoxins; Versicolorin A; Aflatoxin B1; Genotoxicity; Mutagenicity; DNA damage

1. Introduction

Mycotoxins are toxic metabolites produced naturally by various filamentous fungi that frequently contaminate a wide range of agricultural commodities and food products (Bennett and Klich, 2003). The risk associated with exposure to some of these compounds is well characterised and regulations/recommendations have been made concerning their maximum levels in foodstuffs (Payros et al., 2021). However, this is not the case for other mycotoxins, commonly referred to as emerging mycotoxins.

Versicolorin A (VerA) is an emerging mycotoxin that has attracted recent attention by researchers due to its high toxicity. It is a precursor in the synthesis of aflatoxin B1 (AFB1; Fig.1), a mycotoxin known as the most potent natural carcinogen (IARC, 2012; Schrenk et al., 2020). The main fungal species producers of VerA are aflatoxigenic *Aspergillus* species belonging to the section *Flavi (A. flavus* and *A. parasiticus)* and non-aflatoxigenic *Aspergillus* species belonging to the section *Nidulantes*, including *A. nidulans* and *A. versicolor* (Chen et al., 2016; Houbraken et al., 2020). The first group usually contaminates corn, cottonseed, peanuts and walnuts (Marchese et al., 2018), while the second contaminates green coffee beans, spices, nuts, beer, and the outer layer of hard cheese (Díaz Nieto et al., 2018; Gützkow et al., 2022). Exposure to VerA can therefore occur through the consumption of a wide range of foods, but also through inhalation, since *A. nidulans* and *A. versicolor* can grow on materials used inside buildings such as wallpaper, consequently synthesized mycotoxins may be aerosolized from such substrates and enter the respiratory tract (Aleksic et al., 2017).

Much less is known about the toxicity of VerA than that of AFB1, whose presence in food is strictly regulated in most parts of the world due to its notorious dangerousness (European Union, 2006; US Food and Drugs Administration, 2021). AFB1 is a protoxicant that needs to be metabolized by P450 enzymes to become mutagenic (Schrenk et al., 2020). Interestingly, the bisfuran ring that is biotransformed and is responsible for AFB1 toxicity is also present in VerA (Fig. 2). However, while the mutagenicity of VerA is reported in the literature (Mori et al., 1985; Wehner et al., 1978), the importance of biotransformation in VerA mutagenicity has not been confirmed. A few studies have investigated the genotoxicity of VerA at concentrations ranging from 0.1 to 100 µM after 24 h treatment in various cell lines (Budin et al., 2021; Gauthier et al., 2020; Jakšić et al., 2012; Theumer et al., 2018). In intestinal cells, exposure to VerA at 1 µM has been associated with a significant increase in genotoxic markers like γH2AX (Theumer et al., 2018), while higher concentrations of VerA (10 µM) have demonstrated the ability to induce micronuclei and DNA strand breaks (Gauthier et al., 2020). The present paper

aims at providing novel data on the toxicity of VerA. The role of metabolisation in the mutagenicity of VerA was investigated using the SOS/umu and miniaturized Ames test in *Salmonella* Typhimurium strains, with and without metabolic activation. Chromosomal damage and pre-mutagenic lesions were also assessed in p53/P450 competent, non-carcinogenic small intestinal cells using the micronucleus, the standard and the Fpg-modified comet assays, as well as evaluation of DNA damage response proteins using toxin concentrations at least ten times lower than those used in previous studies (starting from 0.01 µM), following two exposure times (6 and 24 h).

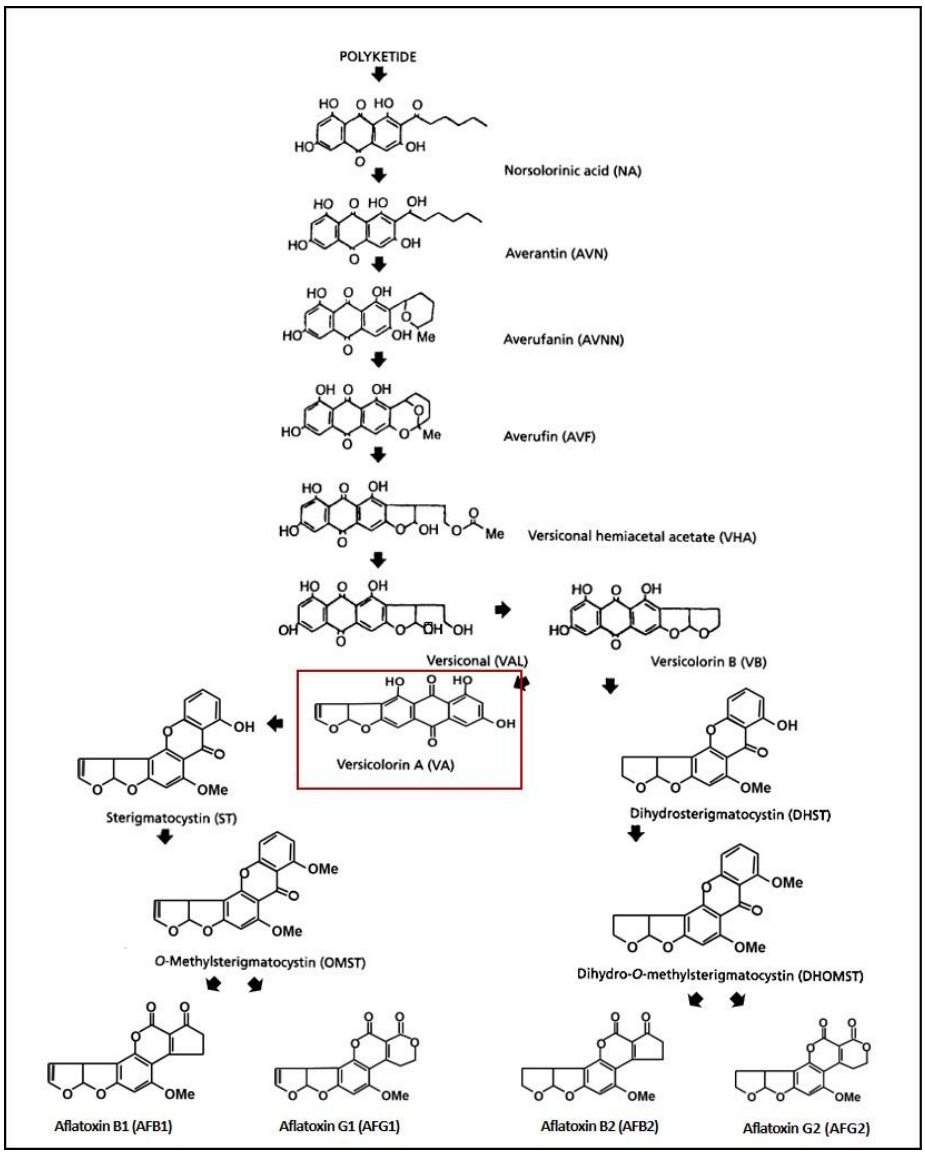

Figure 1: Aflatoxin biosynthetic pathway. *Schematic representation of aflatoxins biosynthesis showing its main intermediates (Adapted from Trail et al.,1995). Versicolorin A is highlighted by the red square.*

Figure 2. Structure of Aflatoxin B1 and Versicolorin A. *The toxic groups are ringed in red, the dihydrobisfuran ring with the double bond for AFB1 in position 8-9 and for VerA in position12-13.*

2. Material and Methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/HAMs F12 medium), phosphate-buffered saline (PBS), insulin transferrin-selenium (ITS), etoposide and DMSO were purchased from Sigma-Aldrich Merck (Saint Quentin Fallavier, France). Analytical grade chloroform, acetic acid and acetonitrile were purchased from Fischer Scientific (Illkirch, France). Streptomycin/penicillin, L-glutamine, fetal bovine serum (FBS) were purchased from Eurobio (Courtaboeuf, France). Epidermal growth factor (EGF) was purchased from Becton-Dickinson (Le Pont de Claix, France). Triton X-100, Methyl methanesulphonate (MMS), potassium bromate (KBrO3), 4,6-diamidino-2-phenylindole (DAPI), HEPES, KCl, 5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma-base, NaOH, Bovine Serum Albumin (BSA), 2-aminofluorene (2-AF), 4-nitro-o-phenylenediamine (NPD), 4-Nitroquinoline 1-oxide (4NQO) and 2-aminoanthracene (2AA) were purchased from Merck (Darmstadt, Germany). Formamidopyrimidine DNA glycosylase (Fpg) was purchased from NorGenoTech (Oslo, Norway). Na_2CO_3 (1M) was purchased from PanReac AppliChem (Barcelona, Spain). Triton X-100 was purchased from Euromedex and Merk (Souffelweyersheim; France; Darmstadt, Germany). Histone H2AX phosphorylated at the C-terminal position on serine 139 (γH2AX), 53 binding protein 1 (53BP1) and the Fanconi anemia group D2 protein (FANCD2) antibodies were purchased from Bio-Techne (Noyal-Châtillon-sur-Seiche, France). Alexa Fluor 488 goat anti-mouse (A32723) and Alexa Fluor 594 anti-rabbit (A32740) were purchased from Life Technologies (Illkirch, France). Goat anti-rabbit secondary antibody and RedDot2 were purchased from Biotium (Montluçon, France). Paraformaldehyde was purchased from Electron Microscopy Science (Pelanne Instruments, France). Rat liver S9 used in the SOS/umu test was purchased from Trinova Biochem (Giessen,

Germany). Mutazyme S9 for the miniaturized Ames test was purchased from Moltox (NC, USA).

2.2. Synthesis and purification of VerA

VerA was self-produced and purified using a previously described in-house procedure (Gauthier et al., 2020). Briefly, VerA was isolated from an *Aspergillus parasiticus* strain (SRRC 0164) grown on wheat grain and purified by Ultimate 3000 high-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, Courtaboeuf, France). The identity and the purity of VerA was configured using HPLC with a diode array detector and a Zorbax C18 analytic column, following a previously established protocol described elsewhere (Theumer et al., 2018). The peak corresponding to VerA represents 98% and 96% of the signal observed at 450 nm and 280 nm, respectively. The concentration of VerA was determined by measuring absorbance at 290 nm (ε25°C EtOH=25,825) and 450 nm (ε25°C EtOH=7,585). The stock solution of VerA was prepared at 10 mM in DMSO and stored at -20 ◦C until further use. Caution was exercised during the production and handling of VerA due to its highly toxic nature and potential health risks. The manipulator utilized a well-ventilated hood and wore double gloves along with a respiratory safety mask during the process

2.3. SOS/umu assay

The mutagenicity of VerA without metabolic activation (PBS), and with liver metabolic activation (8% rat S9 mixture from liver), was tested in *Salmonella* Typhimurium TA1535/pSK1002 (DSMZ 9274, German Collection of Microorganisms and Cell cultures). The SOS/umu assay was performed using the procedure described by Reifferscheid et al. (1991), with the modifications made by Alonso-jauregui et al. (2021). VerA was tested at 84.6 µg/mL and at 15 serial half dilutions. In each assay, DMSO was used as solvent negative control, and 4-NQO and 2AA were included as positive controls in the absence and presence of the S9 mix, respectively. Absorbance at 600 nm (A_{600}) was measured for toxicity where the % survival was calculated using formula 1. β -galactosidase activity was assessed by measuring absorbance at 420 nm (A_{420}) and the induction factor (IF) was calculated using formulas 2, 3 and 4. Two technical replicates were performed for each condition. A tested substance was considered "positive" when the IF was equal to or higher than 2 and bacterial survival higher than 80% (Alonso-jauregui et al., 2021).

$$\%\ survival = \frac{A600\ for\ each\ concentration\ tested}{Average\ A600\ for\ negative\ control} \times 100 \quad (1)$$

$$\beta - galactosidase\ activity\ relative\ units\ (RU) = \frac{A420\ for\ each\ concentration\ tested}{A600\ for\ each\ concentration\ tested} \quad (2)$$

$$Induction\ factor\ (IF) = \frac{RU\ for\ each\ concentration\ tested}{Average\ RU\ for\ negative\ control} \quad (3)$$

$$Average\ RU\ for\ negative\ control = \frac{Average\ A420\ for\ negative\ control}{Average\ A600\ for\ negative\ control} \quad (4)$$

2.4. Miniaturised Ames test

The mutagenicity of VerA was also evaluated with the mini Ames test on two *Salmonella* Typhimurium strains (Moltox, NC, USA), TA98 and TA100, with and without external metabolic activation, using the 6-well plate integration approach previously described elsewhere (Burke et al., 1996; Sanz-Serrano et al., 2021). TA98 and TA100 strains enabled the recovery of all classes of frameshift mutations and base pair substitution mutations, respectively. These two strains were selected to limit the amount of VerA, which is not commercially available, as they enable the detection of most bacterial mutagens (Williams et al., 2019). VerA was tested at five different concentrations (1/3 dilutions) in the range 0.8 of 67.5 µg/well. For both strains in each experiment, 2AF (10 µg/plate) and NPD (20 µg/plate) were used as positive controls, with and without the S9 mixture, respectively. Three technical replicates were performed for each condition. The following criteria were used to assess a compound's ability to cause point mutations: a) the number of revertant colonies per well, whether or not it had a metabolic activation system, if it increased twofold at one or more concentrations in at least one strain, b) if it showed a dose-response relationship over the range tested.

2.5. Cell culture

The choice of the cell system used to test genotoxicity was based on recommendations for the correct evaluation of results. In particular, p53 competent cells are required to reduce the rate of false positives, since these cells maintain genomic stability after genotoxic stress (Kirkland et al., 2007). A metabolically competent cell system is also desirable to detect protoxicants (like AFB1). A small intestinal cell line was preferred because although the toxicokinetics of VerA is not yet known, the molecule is known to be a food contaminant. The non-transformed, non-

cancerogenic intestinal porcine epithelial cell line IPEC-1 was chosen because it is competent for P450 cytochromes and p53 responses. Cells were cultured in DMEM/HAMs F12 medium supplemented with 5% FBS, 1% ITS, 1% L-glutamine, 1% streptomycin/penicillin, and 5 µg/ml EGF. Cultures were kept under controlled conditions in a humidified atmosphere with 5% CO_2 at 39°C.

2.6. Evaluation of cell cytotoxicity and proliferation

Exposure to conditions that compromise cell viability and cell proliferation can potentially result in confounding results in genotoxicity tests due to either non-specific or delayed (thus undetected) DNA damage (Azqueta et al., 2022). In order to ensure a more accurate assessment of genotoxicity and to encompass a broader range of mechanisms of action, the dose-dependent VerA effect in cell cytotoxicity and proliferation was characterized. To estimate cytotoxicity, cell viability of treated and control cells was compared immediately after exposure. To evaluate the effect in cell proliferation, cell viability of treated and control cells were compared after incubation in fresh medium for two cell cycles (48 h) after exposure. A statistically significant loss of cell viability was defined as exceeding 20% (Budin et al., 2021).

To carry out the experiments, IPEC-1 cells were cultured at a density of 5.10^3 cells/ml in a 96-well plate and exposed to increasing concentrations of VerA (ranging from 0.1 to 20 µM) for 6 and 24 h. A real-time cell viability assessment, based on the evaluation of cellular metabolic competence, was conducted using a non-lytic luminescence plate-based method (RealTime-Glo™ MT assay; Promega, G9712) and a multimode microplate reader (TECAN 113 Spark, Männedorf, Switzerland). The analysis was performed continuously and followed the instructions provided by the manufacturer.
To ensure the reliability of the results, three independent experiments with three technical replicates were conducted for each condition. Dose-dependent genotoxic responses were evaluated within the non-toxic range, although exposure conditions that had a cytotoxic effect or an effect on cell proliferation were included in order to complete the interpretation of genotoxicity results.

2.7. Micronucleus assay

A micronucleus assay was performed as previously described (Pons et al., 2021). Briefly, 4×10^4 cells per well were grown on glass coverslips in 24-well plates and exposed to increasing

concentrations of VerA (from 0.01 to 3 μM) for 24 h, a time equivalent to 1.5 – 2.0 normal cell cycle duration. Control cells treated with DMSO were used as negative controls. The ability of the cells to double their cycle was evaluated using a control plate on which the cells were exposed to all the conditions studied and were counted before and after exposure using the Trypan blue exclusion method. For MN analysis, cells were fixed and stained. Three independent experiments were performed for each condition. A minimum of 2 000 nuclei were counted with a Nikon 50i fluorescence microscope equipped with a Luca S camera. The test was considered positive when the concentrations tested showed a statistically significant increase in the number of micronuclei compared to the control.

2.8. Standard and Fpg-modified comet assay

The standard and enzyme-modified comet assay was performed as previously described (Sanz-Serrano et al., 2021), but using the 2-gel/slide format and the Fpg enzyme. Briefly, cells (4×10^4 cells/well) were treated with the test compound, positive control, or solvent (DMSO) for 6 and 24 h. The test compound was tested at five concentrations in a range of 0.1–10 μM according to the results of the cell viability assay (Azqueta et al., 2022). In each experiment, IPEC-1 cells were treated with MMS (0.5 mM; 3 hours), while V79 cells (Chinese hamster lung fibroblast) were subjected to KBrO3 treatment (2 mM; 24 h) as positive controls for the standard and Fpg-modified comet assays, respectively. After cell lysis and washes, the Fpg treatment was performed in nucleoids by adding Fpg enzyme or enzyme reaction buffer to the gels and incubated them for 1 h at 37°C. The comet testing protocol according to Sanz-Serrano et al., 2021 was then applied. Three independent experiments were performed for each condition. Under fluorescence microscopy, 50 comets per gel (100 per condition) were randomly scored using the semi-automatic Comet Assay IV software (Instem). The percentage of tail DNA was used as the comet descriptor, and the median percentage of tail DNA among 100 comets was used to calculate the degree of DNA damage. Net Fpg-sensitive sites were estimated by subtracting the percentage of DNA in the tail acquired after incubation in reaction buffer from the percentage obtained after incubation with the enzyme. The following criteria were used to determine whether a substance can cause strand breaks or oxidized bases: (a) A statistically significant increase in percentage tail intensity at one or more concentrations, (b) a concentration-dependent response.

2.9. Evaluation of the cellular response to DNA damage using γH2AX in-cell Western and immunofluorescence analysis

An overview of the VerA induction of γH2AX at different concentrations for 6 and 24 h was obtained using the medium-throughput In-Cell Western Assay (Khoury et al., 2013). To complete this evaluation, a more sensitive immunofluorescence (IF) analysis of γH2AX, 53BP1 and FANCD2 was carried out.

The In-Cell Western (ICW) assay was performed as previously described (Khoury et al., 2013; Payros et al., 2017). Cells were exposed to increasing concentrations of VerA (from 0.01 to 3 μM) for 6 and 24 h. Control cells were treated with DMSO and etoposide (5 and 10 μM), used as a negative and positive controls, respectively. Cells were then fixed, permeabilised, blocked, and then incubated with primary antibody anti-γH2AX diluted 1:200. The Sapphire Biomolecular Imager (Azure Biosystems) was used for signal acquisition. Three independent experiments and three technical replicates were performed for each condition. To calculate the genotoxic index, the γH2AX signal was divided by the associated DNA fluorescence and normalised with the average fluorescence of untreated control cells. Test results were considered positive when at least one concentration responded above the established induction threshold of 1.2 times with a standard deviation (SD) < 20% and cell viability ≥ 70% (Khoury et al., 2013).

For IF analysis, the cells were pre-extracted with 0.5% Triton X-100 in PBS for 5 min, and the assays were then performed as previously described (Gauthier et al., 2020). Cells were exposed to increasing concentrations (from 0.1 to 3 μM) of VerA and DMSO for negative controls for 24 h. Three independent experiments were performed for each condition. A fluorescent Nikon Eclipse 50i microscope with a 20x objective and equipped with a Luca S camera was used to capture the images. A minimum of 400 cells per condition were imaged and the signal intensity of each nucleus was automatically determined by an ImageJ macro. Statistical differences between control and treated cells were then investigated.

3. Statistical Analysis

Data from SOS/umu and Ames test are expressed as mean ± SD of three technical samples. Data from micronucleus test, comet assay, ICW assay as well as IF analysis are expressed as mean ± SD of three independent experiments. The statistical analysis was performed by a one-way Anova followed by Dunnett multiple comparison tests using GraphPad Prism version 8

software (San Diego, California USA). The difference in a p value ≤ 0.05 (*); ≤0.01 (**); ≤0.001 (***); ≤0.0001 (****) was considered statistically significant.

3. Results

3.1 VerA causes point mutations in the presence of metabolic activation

The SOS/umu was used as a screening test for mutagenicity due to its high concordance with the standard Ames test (TG OECD 471) (OECD, 2020; Reifferscheid and Heil, 1996). DNA damaging agents induced the SOS response in bacteria monitored using colorimetric measurement. The positive controls (4NQO and 2AA) produced the expected increase in mutation frequency (Supplementary data). VerA precipitated at the highest concentration (84.6 µg/mL) and was toxic to the bacteria (42.29 and 21.14 µg/mL) with metabolic activation (Fig. 3, B). These concentrations were consequently not retained for interpretation of data in metabolic activation conditions. In the absence of metabolic activation, the induction factor (IF) of VerA was less than 2 at all concentrations (Fig. 3, A). In contrast, the IF was greater than 2 starting from 0.66 µg/mL in the presence of metabolic activation with a concentration-response trend (Fig. 3, B). Overall, the results showed a positive response by VerA in the SOS/umu assay, but only with metabolic activation.

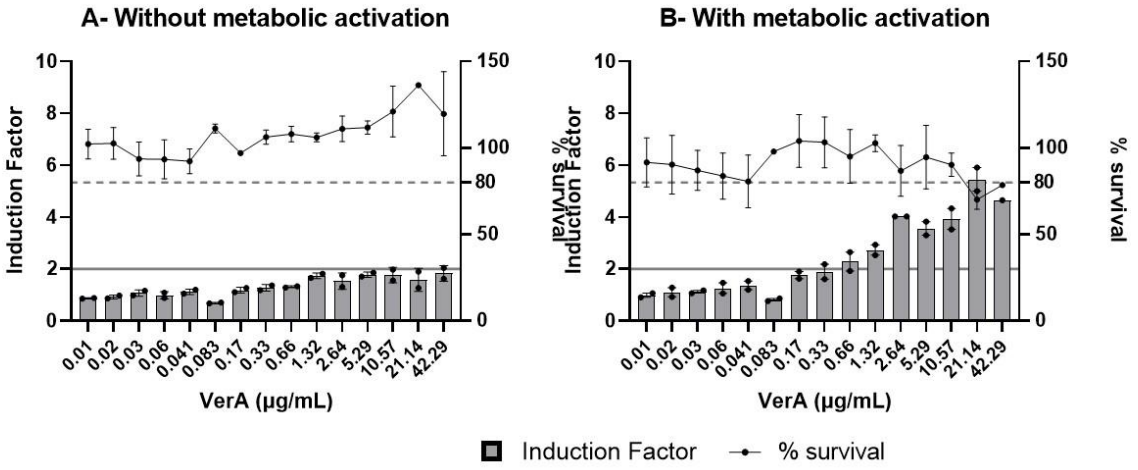

Figure 3: Results of the SOS/umu test for VerA without (A) or with (B) metabolic activation. *Bacterial survival is expressed as a percentage. The mean induction factor (IF) and % of bacterial survival ± standard deviation (SD) of technical duplicates of one experiment are shown. Concentrations are considered non-toxic if bacterial survival is >80%. A compound is considered genotoxic if the IF is ≥ 2*

at non-toxic concentrations for the bacteria in any of the conditions tested. The bars represent the induction factor (IF) and the black line represents the percentage of bacterial survival. The grey line indicates IF = 2 and the dashed grey line indicates % survival = 80%.

Mutagenicity was subsequently assessed using a miniaturised version of the Ames test in two *Salmonella* Typhimurium strains (TA98 and TA100), with and without metabolic activation (S9+/S9-; Table 1). The Ames test, also known as the bacterial reverse mutation test, is used to determine the mutagenic potential of a substance by studying its ability to induce reverse mutations in different hypersensitized bacterial strains. The positive controls (2-AF and NPD) produced the expected increase in mutation frequency and the bacterial phenotypes were confirmed. VerA induced toxicity and was insoluble at a concentration of 67.5 µg/well in both tested strains. In this context, only the lower doses (0.8 - 22.5 µg/well) were used for interpretation. In the absence of S9 activation, two concentrations (2.5 and 7.5 µg/well) of VerA induced a more than two-fold increase in both strains compared to the solvent control, although this response was not dose dependent. In contrast, no two-fold induction of revertant colonies was observed at 0.8 and 22.5 µg/well (toxic concentration) of VerA in either strain tested (S9- ; Table 1). In the presence of metabolic activation, all concentrations of VerA doubled the number of revertants and induced a significant dose-dependent increase in the number of revertant colonies in both strains compared with the solvent control, except for TA100 at 0.8 µg/well. Although the results without metabolic activation were equivocal, VerA was mutagenic with metabolic activation in *Salmonella* Typhimurium strains (TA98 and TA100).

Table 1. Results of the miniaturised Ames test used for VerA in Salmonella* Typhimurium *TA98 and TA100 without (S9-) or with (S9+) metabolic activation. *Mean revertant/plate ± standard deviation (SD) of the technical triplicates of one experiment are shown. Negative (C-: vehicle DMSO) and positive (C+) controls are shown as mean revertant/plate. The positive controls were 20 µg/well 4-nitro-o-phenylenediamine (NPD; S9-) and 10 µg/well 2-aminofluorene (2-AF; S9+). A compound is considered mutagenic if the number of revertant colonies increases twofold in at least one strain and in a dose-response relationship over the range tested. *: toxicity observed (loss of bacterial lawn).*

Concentration VerA (µg/plate)			C- (DMSO)	0.8	2.5	7.5	22.5*	C+ (2-AF/NPD)
Mean revertants / plate ± SD	TA98	S9 -	17 ± 3	32 ± 3	64 ± 6	65 ± 21	41 ± 19	256 ± 6
		S9 +	19 ± 6	138 ± 23	196 ± 18	232 ± 6	89 ± 8	311 ± 21
	TA100	S9 -	34 ± 1	67 ± 3	137 ± 15	91 ± 9	63 ± 10	258 ± 12
		S9 +	53 ± 2	84 ± 6	173 ± 11	204 ± 12	323 ± 27	388 ± 18

3.2 A short period of exposure to low concentrations of VerA induces cytotoxicity and impairs cell proliferation

The cell viability of VerA was evaluated after 6 and 24 h of treatment using the RealTime-Glo™ MT assay. More than 20 % loss of cell viability was considered cytotoxic (Garofalo et al., 2023). Moreover, the effect on cell proliferation was determined after performing the same assay in control and treated cells 48 hours after removing the medium containing VerA and replacing it with fresh medium. Results are shown in Fig. 4. No loss of viability was observed after 6 h of exposure to VerA concentrations up to 6.5 µM in IPEC-1 cells, whereas exposure to 10 and 20 µM produced cytotoxicity (Fig. 4, A). However, VerA affected cell proliferation only in cells treated with 3 µM or higher concentrations (Fig. 4A). After 24 h of exposure, cytotoxicity was observed in cells treated with 6.5 µM VerA and higher concentrations (Fig. 4, B). However, cell proliferation was affected in cells treated with 1 µM VerA and higher concentration. A concentration-dependent response was observed in all cases.

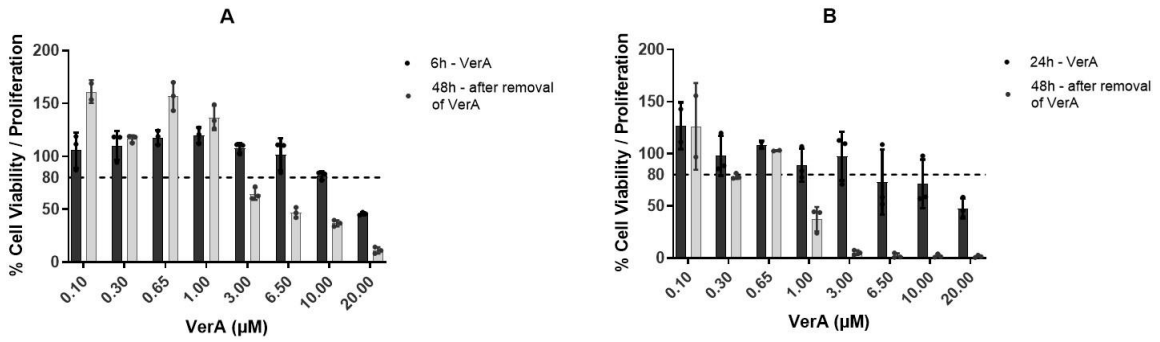

Figure 4. Effects of different concentrations of VerA (µM) on cell viability after 6 and 24 h of exposure and after 48 h of VerA removal in IPEC-1 cells*. Proliferative IPEC-1 cells were incubated with increasing concentrations of VerA for 6 h (A) or 24 h (B). Cell viability was determined immediately after the treatment (black bars) and 48 hours after removing the medium containing VerA and replacing it with fresh medium (cell proliferation; grey bars). Results are expressed as the mean ± SD of 3 independent experiments. The dashed lines indicate % viability = 80%.*

3.3 VerA causes chromosomal damage without affecting cell proliferation ability

The micronucleus (MN) assay detects micronuclei in the cytoplasm of interphase cells and consequently offers a comprehensive basis for studying chromosomal damage *in vitro*. MN formation was measured in our assessment of the mutagenicity of VerA (Fig. 5). VerA

generated micronuclei in cells exposed for 24 h in a dose-dependent manner from 0.1 to 1 μM. We also observed that cells were able to double their cell cycle at all concentrations of VerA except 3 μM (data not shown), which may explain the decrease in VerA-mediated MN induction at this concentration. These results demonstrated that VerA induces clastogenicity and/or aneugenicity in IPEC-1 cells.

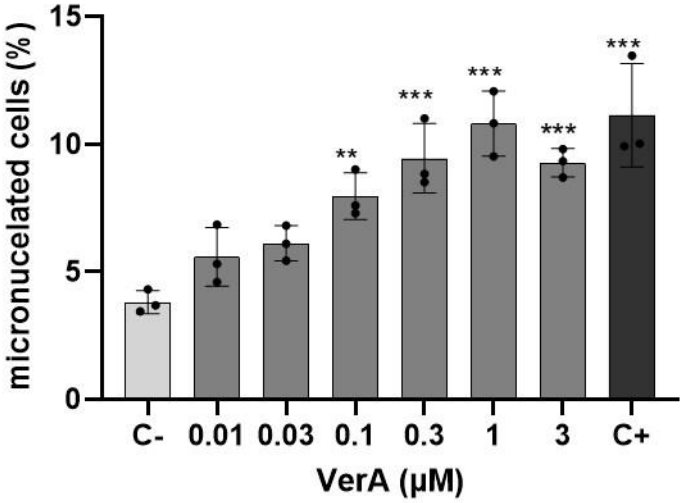

Figure 5. Frequency of micronucleus induction after exposure of IPEC-1 cells to increasing concentrations (μM) of VerA for 24 h*. Cells were treated with mitomycin C (MMC;* 0.1 μM) *in the positive assay control (C+). Micronucleus quantification is expressed as the mean ± SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001 versus CTRL (C-; DMSO), ANOVA followed by Dunnett's post-hoc test).*

3.4 VerA causes pre-mutagenic lesions at low concentrations

The standard alkaline comet assay is used to detect DNA strand breaks (SBs) and alkali labile sites (ALS), whereas the Fpg-modified version is used to detect oxidized bases. In the standard version, VerA showed a significant increase in the percentage of tail intensity at 1 and 3 μM after 6 h of exposure (Fig. 6, A). However, this increase was not observed at 10 μM, thus a concentration-dependent response was not observed. After 24 h of exposure, a significant concentration-dependent increase was observed in cells treated with the highest concentrations tested, 3 and 6.5 μM (Fig. 6, B). However, the latter concentration (6.5 μM) exhibited cytotoxic effects with cell survival below 80%. Results of the Fpg-modified comet assay indicated that VerA induced significant increase in DNA Fpg-sensitive sites in exposed cells starting at 0.3 and 0.65 μM at 6 and 24 h of exposure, respectively, in a concentration-dependent manner.

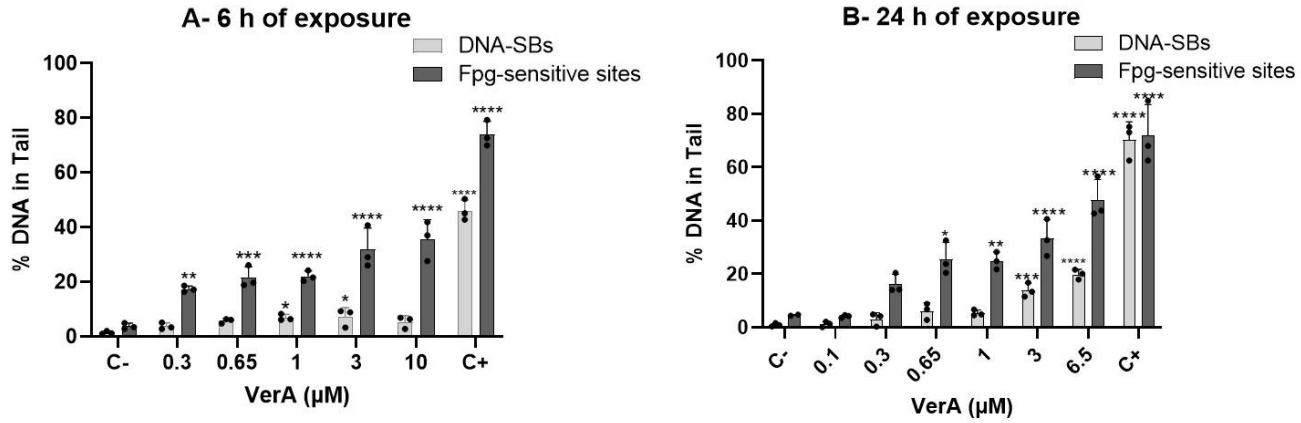

Figure 6. DNA damage evaluated using the standard and Fpg-modified comet assay (+Fpg) after treatment of IPEC-1 cells with increasing concentrations of VerA for 6 h (A) and 24 h (B). *Positive controls (C+) were methyl methanesulfonate (MMS; 0.5 mM for 3h) and potassium bromate ($KBrO_3$; 2 mM for 24 h) for each test, respectively. Negative control was vehicle (DMSO) alone. Results are mean ± SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus CTRL, ANOVA followed by Dunnett's post-hoc test.). DNA-SBs: DNA-strand breaks, Fpg: formamidopyrimidine-DNA glycosylase.*

The different factors involved in the DNA damage response (DDR) were analysed to obtain more mechanistic information on the type of biological damage induced by VerA. The γH2AX ICW assay measures phosphorylation of histone H2AX (γH2AX), a well-known marker of DNA double-strand breaks (DSB). This assay was used to assess DNA damage caused by exposing cells to increasing doses of VerA for 6 and 24 h (Fig. 7). VerA treatment induced a dose-dependent increase in γH2AX fold induction, the lowest effect being observed at concentrations of 0.03 and 0.1 µM after 6 and 24 h of exposure, respectively (FC>1.2). After 24 h of exposure, the results of the cells exposed to 3 µM of VerA were not exploitable and are thus not shown.

To corroborate the ICW results, IF analyses were performed after 24 h of VerA treatment, by observing H2AX phosphorylation and the recruitment to chromatin of 53BP1, a protein that specifically binds to DSB sites (Fig. 8, A, B). A dose-dependent increase in γH2AX or 53BP1 signals was observed starting from, respectively, 0.3 and 0.1 µM of VerA, confirming that VerA induces DSB in exposed cells. Finally, the induction of FANCD2 foci was observed by IF in pre-extracted cells (Fig. 8, C). FANCD2 is recruited to blocked replication forks and is thus a marker of replication stress. Compared to control cells, VerA induced an increase in FANCD2

signal from 0.1 µM, supporting the hypothesis that VerA genotoxic activity results in the induction of replication stress.

Overall, our results demonstrate that VerA causes important DNA damage after 6 and 24 h of incubation.

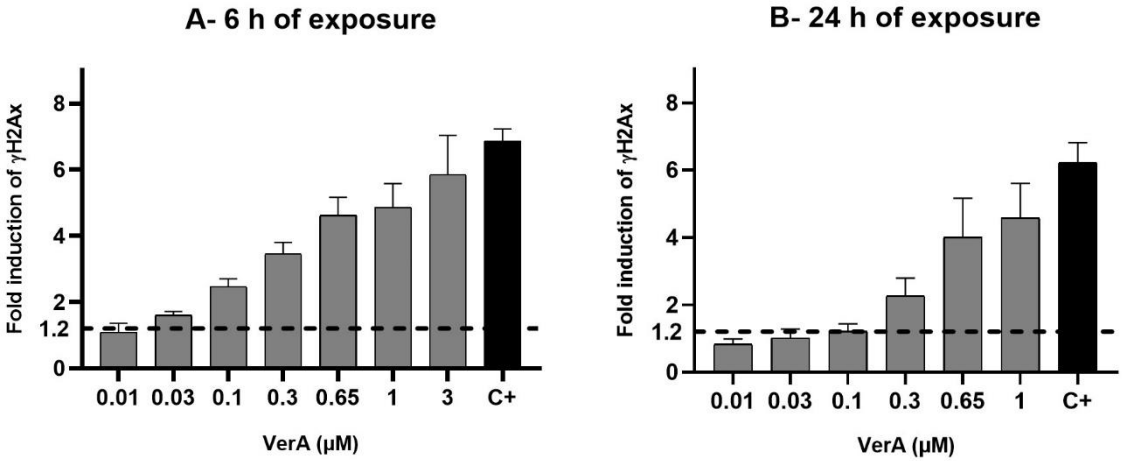

Figure 7. Effect of VerA on H2AX phosphorylation in IPEC-1 cells after 6 (A) and 24 h (B) revealed by the yH2AX-ICW assay*. Cells were treated with etoposide for positive assay control (C+; 5 and 10 µM for 6 and 24 h, respectively). Results are expressed as mean fold induction of yH2AX ± SD of three independent experiments. A compound is considered genotoxic if the induction factor (IF) is ≥1,2 and SD < 20% at non-toxic concentrations for the cells.*

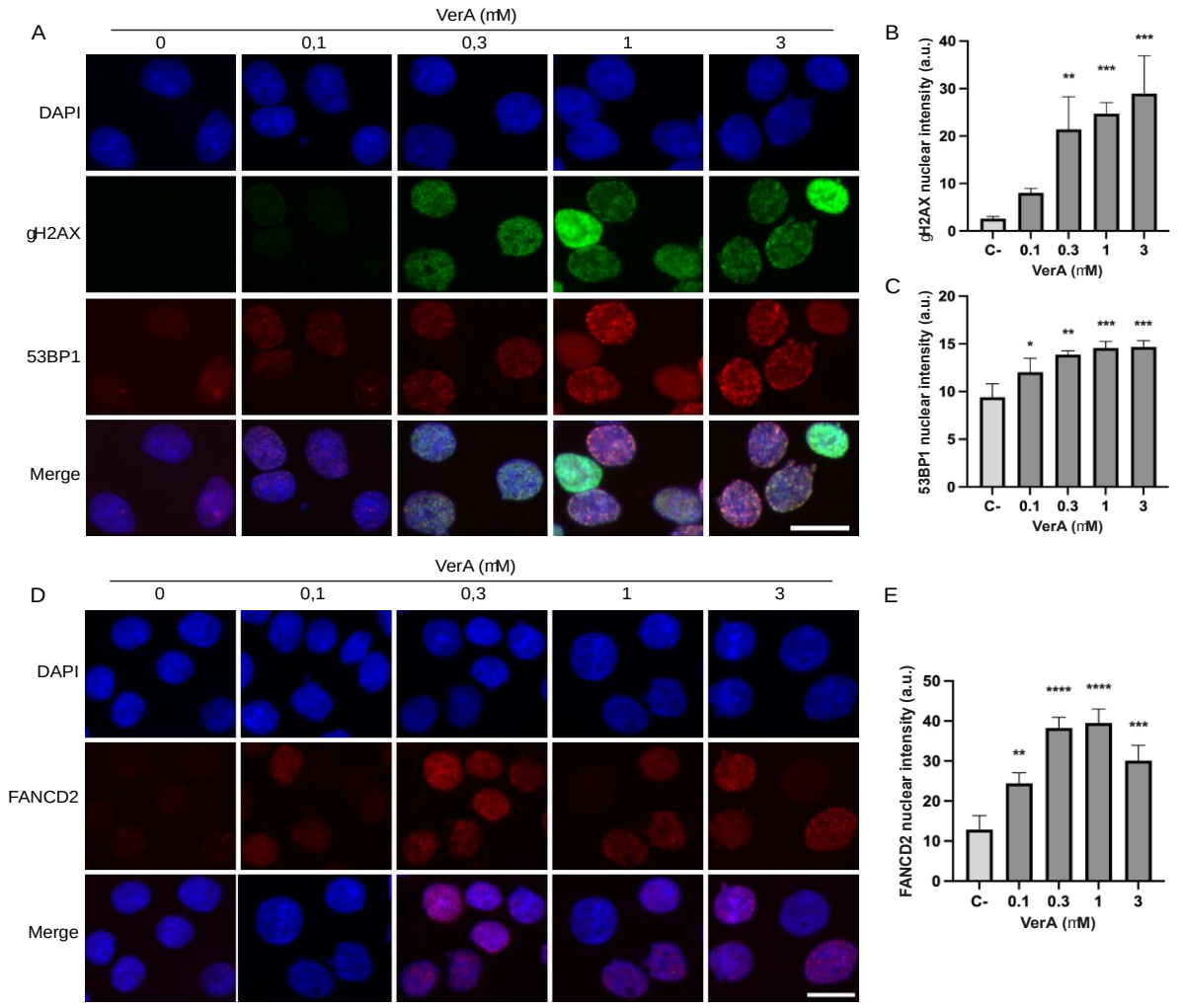

Figure 8. Representative images (A and D) and quantification of the intensity of γH2AX (B), 53BP1 (C) and FANCD2 (E) immunostaining in IPEC-1 cells after 24 h of VerA treatment*. Scale bar = 20 μm. Results are expressed as mean fluorescence intensity per cell of nuclear markers ± SD of the three independent experiments (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different from control cells, ANOVA followed by Dunnett's post-hoc test). γH2AX: histone H2AX phosphorylated on serine 139; 53BP1: p53-binding protein 1; FANCD2: Fanconi anemia group D2 protein.*

4. Discussion

The carcinogenicity of AFB1 depends on a structural feature, an unsaturated bond at the 8, 9 position on the terminal furan ring (Schrenk et al., 2020). Therefore, all AFB1 precursors presenting the same feature, including VerA, are potentially genotoxic and carcinogenic. The production of data to assess the potential toxicity of carcinogenic compounds is a crucial step in the assessment of hazard needed to enable regulations to be made. However, only limited

data on the mutagenicity and genotoxicity of VerA are currently available. In the present work, we established for the first time that metabolization is important for VerA mutagenicity. We also obtained unprecedented results showing that VerA induced chromosomal damage and other pre-mutagenic lesions in intestinal cells after a short period of exposure to non-cytotoxic concentrations of toxin.

Using two complementary assays, the SOS/umu and the miniaturised Ames test, we observed a positive mutagenic response only in the presence of metabolic activation. Ours is the first study to investigate the VerA-dependent induction of point mutations using a wide range of concentrations, using metabolic activation. We can therefore only compare the present findings with the results reported by Wehner et al. (1978), who observed clear mutagenic activity only in the presence of the S9 fraction in TA98 and TA100 while a weaker response was observed without metabolic activation (Wehner et al., 1978). Our data, suggest a relationship between the chemical structure and the mutagenic property of VerA. Indeed, the presence of a bisfuran ring in AFB1, STC and VerA has been shown to be associated with high mutagenicity in TA98 and TA100 strains (Wehner et al., 1978; Wong et al., 1977). The mutagenicity mechanisms of AFB1 and STC are associated with the generation of highly reactive epoxides (AFBO and exo-STC-1, 2-oxide, respectively) through the action of liver cytochrome P450 (CYP) isoforms, which can covalently bind to DNA, leading to point mutations and genetic damage (Rushing and Selim, 2019; Essigmann et al., 1979). Because bioactivation was associated with unequivocal mutagenicity, our results strongly suggest that VerA is biotransformed into a highly mutagenic metabolite similar to that produced from AFB1 and STC (Al-Ayoubi et al., 2022). The identification of VerA-DNA adducts is now needed to confirm this hypothesis.

In our model of small intestinal cells using the micronucleus assay, we observed VerA-associated chromosomal damage at concentrations as low as 0.1 µM after 24 hours of exposure. Interestingly, our results showed a significant effect at concentrations ten times lower than those used in previous studies conducted on pulmonary A549 cells (20 µM for 2 hours; Jakšić et al., 2012) and Caco-2 cells (1 µM for 16 hours; Gauthier et al., 2020). These differences may be explained by the differences in the metabolic and DNA repair competence of these cell lines. Indeed, IPEC-1 cells are non-carcinogenic, p53-competent and express active P450 enzymes, which is relevant in view of the important role of bioactivation in VerA mutagenicity. The formation of micronucleus could be the consequence of chromosome breakage (clastogenicity), loss (aneugenicity) or a combination of both. Other analyses, such as centromere-specific in situ probes are needed to identify the mechanism of VerA-MN induction.

The present results also confirm the high genotoxicity of VerA in intestinal epithelial cells, regardless of the type of pre-mutagenic lesions evaluated. Overall, our results showed unequivocal positive responses in alkaline and modified comet assays at low non-toxic concentrations, compared to previous studies in which a positive result was only observed at concentrations 4 to 10 times higher (Gauthier et al., 2020). VerA is known to induce strong oxidative stress in exposed cells (Gauthier et al., 2020; Muto et al., 1997). This could be linked to DNA oxidation and the formation of 8-oxoguanine, a major endogenous mutagen detected by Fpg in the modified comet assay. Alternatively, the increase in DNA damage after Fpg treatment at such low VerA concentrations could also be also explained by the formation of formamidopyrimidine adducts (VerA-FAPY), which can be recognised by Fpg enzyme, as described for AFB1 (Corcuera et al., 2011). Further studies are needed to understand the importance of these mechanisms in the VerA-dependent induction of DNA damage.

The quantification of several factors involved in the cell DNA damage response (DDR) is emerging as a strategy to assess genotoxicity which provides mechanistic information. Histone H2AX is phosphorylated at the C-terminal position on serine 139 (γH2AX) as the initial step in the recruitment and localisation of DNA repair proteins (Löbrich et al., 2010). The quantification of γH2AX is widely used as a surrogate marker of DSBs and genotoxicity (Garofalo et al., 2023). Indeed, senescence, cell metabolism, oxidative stress, and apoptosis can also induce γH2AX (Schütz et al., 2021). This has led to the use of additional repair proteins such as 53BP1, to confirm the detection of DSBs (Panier and Boulton, 2013). In the present study, a significant dose-response induction of γH2AX and 53BP1 signals was observed in IPEC-1 cells at very low concentrations (0.03 µM and 0.1 µM), indicating the induction of DNA double-strand breaks (DSBs) as demonstrated by ICW and IF analyses. The induction of γH2AX by VerA was also reported in previous studies, although the lowest effective concentration observed was at least 10 times higher (1 µM in LS-174T, HepG2, and ACHN cells (Theumer et al., 2018), 10 µM in HepG2 (Budin et al., 2021) and 1 µM and 10 µM in Caco-2 cells (Gauthier et al., 2020)). Based on our results, this possibly reflects the induction of pre-mutagenic lesions linked with the formation of DNA adducts and/or DNA oxidised bases, but also the VerA-dependent replication stress already described (RS; Gauthier et al., 2020). Indeed, the response of the marker of RS FANCD2 increased significantly upon exposure to VerA. This DDR factor is known to play a central role in the repair of DNA interstrand crosslinks, and is predominantly expressed in highly proliferative cell types (Michael et al., 2003). These results are in agreement with our findings, as we observed the

highest number of FANCD2 foci at a concentration of 1 µM of VerA. According to our results, RS could be the consequence of replication fork blockage at VerA-induced Fpg-sensitive sites if they were related to the presence of VerA-FAPY adducts. A second possibility is that VerA was implicated in transcription disruption, representing a source of RS. The latter hypothesis is supported by the significant accumulation of sequences corresponding to small nucleolar RNAs (both box C/D and box H/ACA snoRNAs) and the impact of VerA on the expression of several genes involved in RNA processing (Gauthier et al., 2020). Taken together, these results suggest that the induction of RS plays an important role in the genotoxic activity of VerA, and could explain the VerA-mediated induction of γH2AX in cell lines devoid of metabolic activity, such as ACHN cells (Theumer et al., 2018).

5. Conclusions

The present study showed that the emerging mycotoxin VerA is highly genotoxic, inducing point mutations, chromosomal damage and various pre-mutagenic lesions on intestinal cell lines metabolically competent at concentrations lower than those previously reported in the literature. Our results strongly suggest that like AFB1, VerA may be able to form DNA adducts following metabolic activation, but also that oxidative damage and RS may play an important role in its genotoxicity. Because VerA induced DNA damage at very low levels which did not affect viability, cells bearing mutations can propagate, suggesting a high carcinogenic potential for VerA that calls for further research (Schrenk et al., 2020). The present results contribute to efforts highlighted by EFSA suggesting more toxicity data should be produced on AFB1-related molecules by following their recommended genotoxicity testing strategy, which comprises a core battery of in-vitro assays, including Ames and micronucleus assays, together with other tests to assess primary DNA damage (EFSA, 2011). According to occurrence data (Gauthier et al., 2020), the contamination of VerA can be quite high in some foodstuffs and AFB1-contaminated samples also contained VerA. Here, we showed significant toxicity following exposure to contamination levels ten times lower than previously expected, thereby providing valuable data for risk assessment. Indeed, our results as in the case of AFB1, point to a potential genotoxic and carcinogenic mode of action, suggesting that no Health Based Guidance Value could be established as any level of exposure could theoretically lead to cancer. Our results also provide mechanistic information that will be useful to evaluate interactions between AFB1-related molecules.

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