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Abstract

Aflatoxins are found as food contaminant and some of them demonstrate a carcinogenic effect. The aflatoxins biosynthetic pathway involves 15 successive steps. The aim of this study was to compare the toxicity of afla- toxins and their precursors in three human cell lines. We tested the four aflatoxins and two of their metabolites; three early metabolic precursors and two late biosynthetic precursors. Cyclopiazonic acid, synthesized in parallel with aflatoxins, was also tested. The cytotoxicity and the genotoxicity was evaluated with the γH2AX assay in three human cell lines with different bioactivation capacities. Our results indicated that the most genotoxic chemicals in the three cell lines were in decreasing order sterigmatocystin (ST), aflatoxin B1 (AFB1), aflatoxicol (AFL), aflatoxin G1 (AFG1) and versicolorin A (VERA). Aflatoxin M1 (AFM1) demonstrated genotoxic property in only one cell line. The other tested compounds did not demonstrate any genotoxic activity. Overall, our results suggested different genotoxic mechanisms of action for the tested compounds, involving specific bioactivation pathways. Moreover, some metabolic precursors of aflatoxins demonstrated genotoxic potential equivalent or greater to AFB1. This should be taking into account for the development of new strategies intended to reduce the aflatoxins exposure and for human risk assessment.

Introduction

Aflatoxins are fungal secondary metabolites produced by several Aspergillus species from the flavi section which infect several food commodities (Paterson and Lima, 2010). Aflatoxins constitute a group of closely related compounds, that have strong detrimental impact on the public health and the economy (Kensler et al., 2011). Aflatoxin B1 (AFB1) is the most potent naturally occurring carcinogen reported to date (classified as Group 1 by IARC), having hepatotoxic, immunotoxic and teratogenic properties (IARC, 2012). The most important target organ of AFB1 is the liver (Meissonnier et al., 2007), where the toxin is metabolized principally by CYP1A2 and CYP3A4 resulting in DNA ad- ducts formation (Oda et al., 2001) and cause numerous mutations, notably in the p53 tumor suppressor gene (Eaton and Gallagher, 1994; Kensler et al., 2011). Moreover, prostaglandin H synthase could also metabolize AFB1 in a mutagenic chemical (Battista and Marnett, 1985). AFB1 has also been reported to induce an oxidative stress in vivo (Guindon et al., 2007) as well as in vitro (Parveen et al., 2014), independently of enzymatic bioactivation. However, it is important to recall the probably interactive effects of some

well documented AFB1 co-occuring risk factors, such as chronic hepatitis B and C infections, in the hepatocellular carcinoma (HCC) development (Hamid et al., 2013).
The current knowledge on the aflatoxins production indicates that at least 30 genes are involved in the aflatoxins biosynthesis and are clustered within a 75 kb region of the fungal genome located roughly 80 kb away from telomere (Yu, 2012). About fifteen intermediates form the entire aflatoxin biosynthetic pathway (Fig. 1) and each of them could be found in contaminated food and feed (Streit et al., 2013; Varga et al., 2013). Several strategies have been developed to limit the AFB1 exposure (Holmes et al., 2008; Wu and Khlangwiset, 2010). They include resistant crops, agricultural practices management, and the use of microorganisms, natural products or chemicals that alter known environmental and physiological modulators of aflatoxins biosynthesis, or they alter signaling transduction pathways in the upstream regulatory network. Nevertheless, attempts to reduce the food contamination with aflatoxins may also induce the accumulation of any/several aflatoxin intermediates, or even other metabolic pathway products such as the cyclopiazonic acid (CPA), whose toxicities were not fully explored yet, notably in human cells. Strategies that aimed to interrupt its bio- synthesis lead us to wonder about the toxicity of these intermediates or even other metabolic pathway product like cyclopiazonic acid.
Most toxicity studies only focused on some aflatoxins or on its hydroxylated metabolite (AFM1). We previously demonstrated, using human cell lines with distinct biotransformation properties, the efficiency of the γH2AX ICW genotoxic assay to determine the genotoxic potential of AFB1 (Khoury et al., 2013; Khoury et al., 2016b). The aim of the present study was to compare the cytotoxicity and the genotoxicity potential of twelve molecules: the aflatoxins and their meta- bolites (AFB1, AFB2, AFG1, AFG2, AFM1 and aflatoxicol (AFL)); five intermediates: the sterigmatocystin (ST), the O-methylsterigmatocystin (OMST), the averantin (AVE), the norsolorinic acid (NOR) and the versicolorin A (VERA); and one co-metabolite: the cyclopiazonic acid (CPA). The γH2AX ICW assay was used to simultaneously determine cytotoxicity and genotoxicity of these molecules. Three human cell lines, with a wild type p53 status, derived from target organs of mycotoxins were used. Two cell lines expressing phase I and II bioactivation capabilities (LS-174T (colon) and HepG2(liver)), and one with very poor general bioactivation property (ACHN (kidney)) (Khoury et al., 2016b) to highlight we link between the biotransformation process of a chemical and its genotoxic potential.

Materials and methods

Chemicals

AFB1, AFB2, AFG1, AFG2 and AFM1; ST, OMST, and CPA were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Averantin was provided by BioViotica Naturstoffe GmbH (Dransfeld, Germany). AFL was purchased from Fermenteck (Jerusalem, Israel). NOR and VERA were purified from wheat inoculated with Aspergillus toxigenic stocks, as described below. Almost all stock solutions of the compounds were prepared in 100% dimethyl sulfoxide (DMSO). From the stocks, 10-fold dilution series were prepared. Penicillin, strepto- mycin, trypsin, PBS, RNAse A, and Triton X-100 were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The phosphatase in-hibitor cocktail tablets (“PHOSSTOP”) were purchased from Roche (Meylan, France), and the blocking solution (MAXblock Blocking Medium) was purchased from Active Motif (Rixensart, Belgium). CF770 antibody and RedDot2 were purchased from Biotium (Hayward, California, USA). All solvents used in the extraction and high performance liquid chromatography (HPLC) analysis were analytical grade and purchased from Fisher

Scientific (Illkirch, France). Water for HPLC- DAD and molecular biology procedures was purified by using a Millipore MilliQ purification system.

Fungal strains

A. nidulans RAV Pyro 2, a highly NOR producer mutant, was kindly provided by Pr. AM Calvo, Department of Biological Science, Northern Illinois University, DeKalb, Illinois, USA. The *A. parasiticus* SRRC 0164, a highly VERA producer mutant, was kindly provided by S. Beltz, Southern Regional Research Center, Agricultural Research Service/ United States Department of Agriculture, New Orleans, USA.

Production, isolation and purification of NOR and VERA

For NOR production, *A. nidulans* RAV Pyro 2 was cultivated on solid 1% oat meal agar. The inoculated plates were incubated for 7 days at 37 °C, and then the mycelium was harvested and extracted overnight with chloroform. The chloroformic extract was filter-clarified thanks to Whatman® 1PS phase separator filter papers (Whatman, GE Healthcare, Kent, UK) and evaporated to dryness in a rotary evaporator and then with a Zymark TurboVap (McKinley Scientific, Sparta, NJ, United States) with dry nitrogen. The residue was kept at −20 °C until NOR purification by chromatography. VERA was obtained from wheat fermented by *A. parasiticus* SRRC 0164. Briefly, the mutant strain was grown in potato dextrose agar (PDA) plates for 7 days at 28 °C, and thereafter small portions of the developed medium were transferred and dispersed in 14cm diameter Petri dishes containing autoclaved wheat with 35% humidity. The inoculated plates were incubated for 7 days at 28 °C. At the end of the incubation period, wheat and mycelium were harvested and extracted overnight with chloroform. The chloroformic extract was filter-clarified and evaporated to dryness as previously described. The residue was kept at −20°C until VERA purification by chromatography.

The purification of NOR and VERA was performed with an Ultimate 3000 HPLC system (Dionex/ThermoScientific, Courtaboeuf, France). A Strategy C18-2 semi-preparative column was used (250mm length, 7.8mm internal diameter and 5 µM particular size (Interchim, Montluçon, France). The purification of the two compounds was achieved by gradient elution using 0.2% acetic acid (eluent A) and acetonitrile (eluent B) as mobile phase at a flow rate of 2.4 mL/min for NOR and at a flow rate of 4.2 mL/min for VERA. The preparative flow was pumped through a valve at the ultimate 3000 Fraction Collector (Dionex/ThermoScientific).

For NOR purification, the elution started with 50% solvent B over 10 min. The solvent B part increased then to 90% within 5 min. After a 15min isocratic elution, the gradient decreased to the initial value within 5min and remained constant for the last 10min. For VERA purification, the elution started with 46% solvent B over 10 min. The part of the solvent B increase then to 50% within 14 min and increase again to 90% within 4 min. After a 5 min isocratic elution gradient decreased to the initial value within 5 min and remained constant for the last 5 min. For the two molecules, multiple fractions were pooled, and the solvent was evaporated under reduced pressure. Prior to toxi- city experiments, the identity and purity of the purified metabolites were confirmed by several methods described below.

HPLC-DAD analysis

HPLC-DAD analysis was performed on the same apparatus described above. A Zorbax C18 analytic column was used (150mm length, 4.6 mm internal diameter and 5 µM particular size, Interchim). In order to check the norsolorinic acid purification a gradient program

was used with 0.2% acetic acid (eluent A) and acetonitrile (eluent B) as mobile phase at a flow rate of 2.4 mL/min. The elution started with 50% sol- vent B over 10 min, then the gradient increased to 90% within 5 min. After 15 min isocratic elution, the gradient decreased to 50% within 5 min and remained constant for the last 10 min. The presence of NOR was monitored at a wavelength of 305 nm and 463 nm. For the verification of the VERA purification a gradient program was used with the same solvents and the following elution conditions were used: 0–8 min 45% B (flow 1.6 mL/min), then the flow rate decreased to 1.4 mL/min and the solvent B part decreased to 35% within 4 min. After 48 min isocratic elution, the flow rate increased to 1.6 mL/min and the gradient increased to the initial value within 4 min and remained constant for the last 6 min. The presence of VERA was monitored at a wavelength of 287 nm.

High-resolution mass spectrometry analyses

The identity of the two purified compounds was confirmed by liquid chromatography coupled to a high resolution mass spectrometer LTQ Orbitrap XL (Thermo scientific, San Jose, CA, United States), and fitted with an electrospray ionization mode according to Cano et al. (2013). A reverse phase 150 × 2.0 mm Luna C18 (2) column (Phenomenex, Torrance, CA, United States) was used. Twenty µL of methanol dilution of each purified compound were injected directly in the LC system. The flow rate was 0.2 mL/min. the gradient chromatography was performed with 0.1% acetic acid (eluent A) and acetonitrile (eluent B) as mobile phase. The elution started with a linear gradient ranging 20% to 50% for 30 min. Then the solvent B part increased to 90% within 5 min. After a 10 min isocratic elution, the gradient was decreased to initial value within 5 min and remained at this value for the last 10 min. Electro- spray ionization was performed at 4.5 kV. The temperature and voltage were set respectively to 350 °C and 40 V. Resolution was set to 60 000 for m/z range set to 50–800.

Nuclear magnetic resonance analyses

^{1}H and two-dimensional nuclear magnetic resonance (2D-NMR) spectra were obtained on a Bruker DRX-600 Avance NMR spectrometer (Bruker, Wissembourg, France) operating at 600.13 MHz for ^{1}H re- sonance frequency, using an inverse detection 5mm 1H-13C-15N cryoprobe in $CDCl_3$ solution (70–200 µg sample/600 µL solvent in a 5 mm NMR tube). To confirm the chemical structures, samples were analyzed using 2D-NMR including gradient selection (gs)-correlation spectroscopy (COSY), (gs)-heteronuclear single quantum coherence (HSQC) and (gs)-heteronuclear multiple bonding connectivity (HMBC). ^{13}C chemical shifts were determined from the f1 projection of HSQC and HMBC diagrams, because the low amount of product precluded the direct measurement of a carbon spectrum.

Norsolorinic acid exhibited the following properties: ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 7.19 (1H, s, H-4); 7.14 (1H, d, J = 2.5 Hz, H- 5); 6.53 (1H, d, J = 2.5 Hz, H-7); 3.12 (2H, t, J = 7 Hz, H-12); 2.18 (2H, t, H-13); 1.45 (2H, m, H-14); 1.25 (2H, m, H-15); 0.88 (3H, t, H-16). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 164.5 (C-6); 163.0 (C-3); 134.5 (C-4a); 134.2 (C-5a); 121.5 (C-2); 108.6 (C-4); 108.5 (C-1a); 108.3 (C-8a); 108.2 (C-5); 107.4 (C-7); 43.5 (C-12); 30.2 (C-13); 22.5 (C-14); 21.3 (C- 15); 13.8 (C-16); and HR-MS (negative ESI): m/z 369.09785 $(M-H)^-$ (calculated exact mass: 369.0980; deviation (ppm) −0.342). For VERA, HRMS and NMR data were in agreement with the data previously published (Jaksic et al., 2012).

Cell lines and cultures

HepG2 human hepatoblastoma cells (ATCC No. HB-8065), ACHN human renal cell adenocarcinoma cells (ATCC No. CRL-1611), and LS- 174T human epithelial colorectal adenocarcinoma cells (ATCC No. CL- 188) were cultured in αMEM, 10% fetal calf serum v/v, penicillin effective concentration (LEC) for γH2AX induction in each cell line (Audebert et al., 2012). AFB1 was choice as the reference compound for GEF determination (set to 1).

γH2AX in-cell western (ICW) assay

The γH2AX In-Cell Western technique was performed as previously described (Khoury et al., 2013; Khoury et al., 2016a,b; Quesnot et al., 2016). Briefly, HepG2, ACHN and LS174-T cells were seeded 16 h prior to treatment at a density of 3.2 × 104 cells per well in 96-well micro- plates, exposed to chemicals for 24 h, fixed with 4% paraformaldehyde (Electron Microscopy Science, Pelanne Instruments, France) in Phosphate Buffered Saline (PBS) and permeabilized with 0.2% Triton X-100. Cells were then incubated in blocking solution (MAXblock Blocking Medium supplemented phosphatase inhibitor PHOSSTOP and 0.1 g. L-1 RNAse A) prior to 2h incubation at room temperature with rabbit monoclonal anti-γH2AX (Clone 20E3, Cell signaling) primary antibody in PST buffer. Detection was carried out with an infrared fluorescent dye conjugated to goat antibody (CF770, Biotium). For DNA labeling, RedDot2 (Biotium) was added simultaneously to the secondary anti- body. After 1 h of incubation, the fluorescence was measured using an Odyssey Infrared Imaging Scanner (Li-CorScienceTec, Les Ulis, France). The fluorescence corresponding to γH2AX and co-localizing with RedDot2 was integrated and expressed as fold change compared with negative controls. Cell viability was calculated by Relative cell count [RCC or final cell count (treated)/final cell count (control) × 100] assessed by automated fluorescence. All experiments were performed at least three times independently.

Data analysis

Genotoxicity was considered positive when a compound produced a statistically significant 1.5-fold γH2AX induction at level of cytotoxicity below 50% compared to the control. These parameters were based on our previous studies (Khoury et al., 2013; Khoury et al., 2016a,b; Quesnot et al., 2016) and are similar to those used by other groups who use γH2AX quantification (Ando et al., 2014; Bryce et al., 2014; Smart et al., 2011). Error bars represent the standard error of the mean (SEM). Statistically significant increases in H2AX phosphorylation after treatment were compared with controls using Student's test (*, $p < .05$; **, $p < .01$).

Genotoxic equivalent factor (GEF) determination

Genotoxic Equivalent Factors (GEF) for aflatoxins and their pre- cursors was determined based on the comparison of their lowest

Results

First, we examined the cytotoxicity and genotoxicity of the four aflatoxins and two of their metabolites in the three selected human cell lines (Fig. 2). AFB1 and AFG1 demonstrated a genotoxic potential in all the cell lines tested with different potencies. The lowest effective con- centration (LEC) for AFB1 in HepG2, LS-174T and ACHN cells were 1, 0.1 and 10 μM, respectively (Fig. 2A). For AFG1, the observed LEC were 10, 1 and 100 μM in HepG2, LS-174T and ACHN, respectively (Fig. 2B). These two compounds demonstrated

some cytotoxicity at high concentrations, notably in the LS-174T cell line. AFM1 was genotoxic only at the highest concentration tested (10 μM) and only in the LS-174T cells (Fig. 2F) without any sign of cytotoxicity. AFL was genotoxic without any sign of cytotoxicity in HepG2 and LS-174T cells with a LEC of 10 and 0.1 μM, respectively (Fig. 2E). We observed that AFB2 and AFG2 were not cytotoxic nor genotoxic whatever the cell line tested (Fig. 2C and 2D). Based on these results the genotoxic potencies of aflatoxins were in the following order: AFB1, AFG1 and aflatoxicol > AFM1.
Then we examined the cytotoxic and genotoxic potential of the five aflatoxins precursors and one co-metabolite in the three selected human cell lines (Fig. 3). OMST, AVE, NOR and CPA were cytotoxic in the three cell lines at the highest concentration tested (100 μM) but did not demonstrated any genotoxic potential (Fig. 3B, 3D, 3E and 3F). ST and VERA were genotoxic in all the cell lines tested with different potencies. For ST, a LEC of 0.1 μM was observed in LS-174T cells and 1 μM in HepG2 and ACHN cell lines (Fig. 3A). For VERA, the observed LEC was 1 μM whatever the cell line tested (Fig. 2C). VERA demonstrated also high cytotoxicity notably in LS-174T and ACHN cells. Based on these results the genotoxic potencies of aflatoxins precursors were in the following order: ST > VERA.

Discussion

Aflatoxins are frequently found as food contaminant and some of them demonstrate a carcinogenic effect. The aflatoxins biosynthetic pathway lead to different precursors. Strategies to limit the exposure to aflatoxins may lead to the accumulation of these compounds. However, the genotoxic potential of these chemicals has not been studied completely in a human cellular background. For this reason, we performed a strict comparison of the cytotoxic and the genotoxic potentials of twelve aflatoxins and their precursors using the γH2AX ICW assay in three human cell lines with different bioactivation properties. We observed that the LS-174T cell line was the most sensitive cells with seven chemicals detected genotoxic and with the lowest LEC, as low as 0.1 μM for ST, AFL and AFB1, compared to the other cell lines. Conversely, in the ACHN cells, only four compounds were detected genotoxic and with relative high LEC (AFB1, AFG1, ST and VERA).
We determined the genotoxic equivalent factor (GEF) for each of the tested chemicals compared to AFB1 in each cell line tested (Table 1). This concept of GEF was already applied to other carcinogenic food contaminant like polycyclic aromatic hydrocarbon (Audebert et al., 2012). We observed that all the compounds genotoxic with the γH2AX assay in the LS-174T cell line were also carcinogenic, confirming the good predictivity of this assay (Khoury et al., 2016b). We noted that for some compounds, GEF between cell lines was unrelated, notably be- tween HepG2 and LS-174T cells on one hand and ACHN cells on the other hand. Difference in the bioactivation capacities of the cell lines used may account for this observation. ACHN cells have very low phase I and II metabolism capabilities (Khoury et al., 2016b). This characteristic may explain the poor genotoxic potential of AFB1, AFG1 and AFL in this cell line. Indeed these later toxins need CYPs bioactivation to induce DNA damage (Oda et al., 2001). The bioactivation of these compounds by the prostaglandin H synthase (Battista and Marnett, 1985) and/or the induction of an oxidative stress (Guindon et al., 2007; Parveen et al., 2014), may account for the genotoxicity of these aflatoxins in this specific cell line devoid of an important CYPs bioactivation capacity. Conversely, the higher bioactivation capacity of LS-174T compared to HepG2 cells may explain that AFM1 was only genotoxic in the colon cell line. This particularity may be notably related to the phase II enzyme capabilities of the LS-174T cells compared to HepG2 cells. We have previously demonstrate that the 2-amino-

1-methyl-6- phenylimidazo[4,5-b]pyridine (PhIP) heterocyclic aromatic amine, a chemical that need CYP1A2 and SULT1A1 bioactivation to form a DNA reactive metabolite, was only genotoxic in LS-174T and not in HepG2 cells (Khoury et al., 2016b). Additional experiments with specific engineered cell lines with particular bioactivation properties may allow testing this hypothesis of a possible bioactivation of AFM1 by CYP1A2 and SULT1A1 (Chevereau et al., 2017). Overall, our results suggested different independent genotoxic mechanisms of action for the aflatoxins, involving specific bioactivation pathways. The principal pathway implicated bioactivation of aflatoxins by CYP1A2 or CYP3A4 in a DNA reactive metabolite, as in HepG2 and LS-174T cells. The second pathway may implicated in specific bioactivation of aflatoxins by CYP1A2 and SULT1A1 as in the case of AFM1 in LS-174T cells. The last pathway may incriminate the bioactivation of aflatoxins by the prostaglandin H synthase and/or the induction of an oxidative stress like in ACHN cells. These pathways may not be exclusive from each other's and for some compounds multiple pathways may account for their genotoxic potential, depending of the bioactivation properties of the cells.

Concerning the aflatoxins precursors, we noted that OMST, NOR, AVE and CPA were devoid of genotoxic potential whatever the cell line used. These results are in agreement with some previous studies in bacteria with the Ames assay for OMST (Wehner et al., 1978), CPA (Kuilman-Wahls et al., 2002; Sabater Vilar et al., 2003; Wehner et al., 1978) and NOR (Mori et al., 1985; Wong et al., 1977). VERA demonstrated an important genotoxic potential, with the same LEC of 1 µM in all cell lines and a GEF of 0.1, 1 and 10 in LS-174T, HepG2 and ACHN cells, respectively. This observation corroborated results from a mi- cronucleus study in A549 cells (Jaksic et al., 2012) and a hepatocyte/ DNA repair test (Mori et al., 1984). However, in our study VERA genotoxicity was observed whatever the cell line used, suggesting that VERA may be genotoxic independently of its bioactivation by CYPs. VERA, like AFB1, has been observed mutagenic in the Ames assay in absence of exogenous bioactivation system (Wehner et al., 1978). Inhibition of the mitochondria respiratory chain resulting in an oxidative stress had also been notice in human cells with this chemical (Kawai et al., 1983). ST demonstrated a GEF of 1 in LS-174T and HepG2 cells and 10 in ACHN cells. This important genotoxic potency based on γH2AX assay is 10 fold higher than calculated from results with the Ames assay (McCann et al., 1975; Wong et al., 1977) but in accordance with a genotoxic study in human A549 cells (Jaksic et al., 2012). ST and VERA were the only chemicals to exhibit a GEF of 10 in ACHN cells. This feature may be linked to the sensitivity of this cell line to oxidative stress (Khoury et al., 2016b) and the possible inhibition of the mitochondria respiratory chain by VERA (Kawai et al., 1983) and ST (Kawai et al., 1986).

In conclusion, our data demonstrated the similar or greater genotoxic potential of some aflatoxin precursors compared to AFB1 in human cells and their probable carcinogenic capacity. This should be taking into account for the development of new strategies intended to reduce the aflatoxins exposure and for human risk assessment to mycotoxins.

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Table 1
Comparison of the in vitro genotoxic potential of the aflatoxins and their precursors in the γH2AX In-Cell Western assay with their carcinogenic potential. Genotoxic equivalent factor (GEF) were derived from the lowest genotoxic concentration observed

Compounds	GEF LS-174T[a]	GEF HepG2[a]	GEF ACHN[a]	Carcinogenesis[b]
AFB1	1	1	1	+
AFB2	-	-	-	-
AFG1	0.1	0.1	0.1	+
AFG2	-	-	-	-
AFM1	0.01	-	-	+
AFL	1	0.1	-	+
ST	1	1	10	+
OMST	-	-	-	ND
VERA	0.1	1	10	+
NOR	-	-	-	ND
AVE	-	-	-	ND
CPA	-	-	-	ND

[a] Genotoxic Equivalent Factor (GEF) derived from results observed in this study. [b] Data from peer reviewed published articles and from the Carcinogenic Potency Database. Definitions: (ND) “not determined”, (+) tested “positive”, (-) tested “negative”.

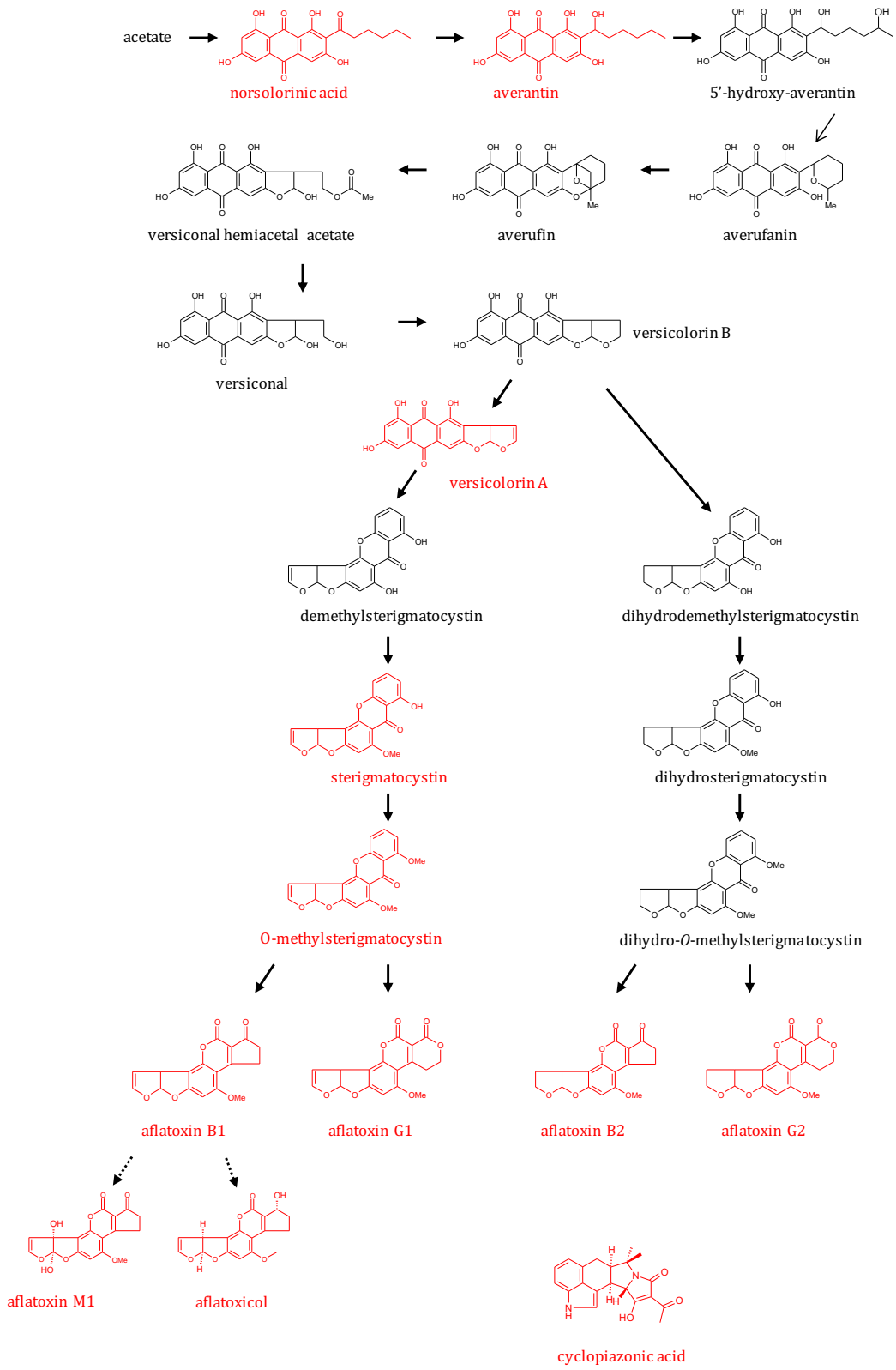

Fig. 1. Aflatoxins biosynthetic pathway. In red were presented the compounds tested in this study.

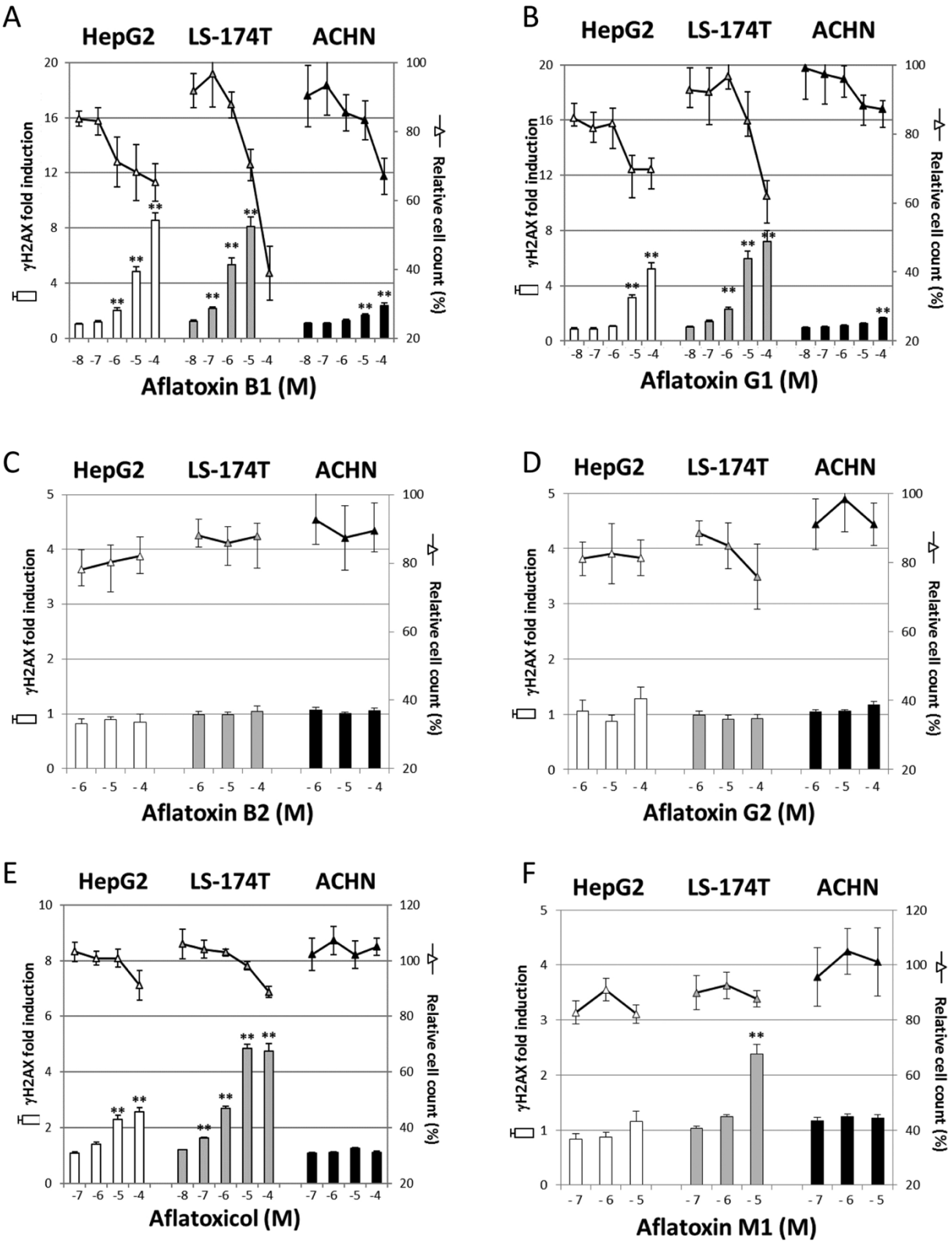

Fig. 2. In vitro cytotoxicity and genotoxicity of aflatoxins with the γH2AX ICW assay in HepG2, LS-174T and ACHN cell lines; aflatoxin B1 (A), aflatoxin G1 (B), aflatoxin B2 (C), aflatoxin G2 (D), aflatoxicol (E) and aflatoxin M1 (F). Each value represents the mean ± SEM (n ≥ 3) after 24 h of treatment. Statistically significant increase in H2AX phosphorylation compared with DMSO control; *, $p < .05$; **, $p < .01$.

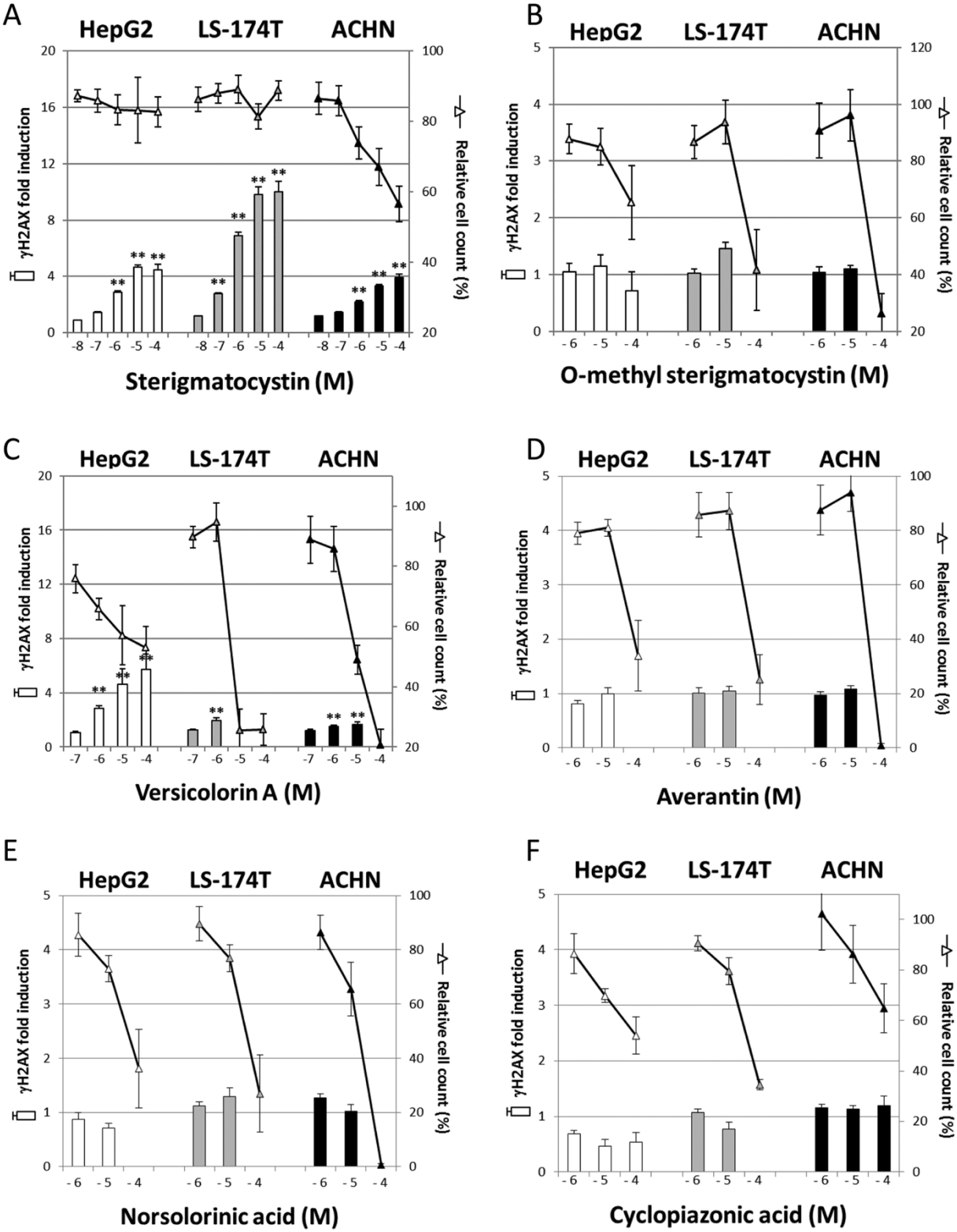

Fig. 3. In vitro cytotoxicity and genotoxicity of aflatoxins precursors with the γH2AX ICW assay in HepG2, LS-174T and ACHN cell lines; sterigmatocystin (A), O-methyl sterigmatocystin (B), versicolorin A (C), averantin (D), norsolorinic acid (E) and cyclopiazonic acid (F). Each value represents the mean ± SEM (n ≥ 3) after 24 h of treatment. Statistically significant increase in H2AX phosphorylation compared with DMSO control; *, $p < .05$; **, $p < .01$.