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Assessing the combined toxicity of the natural toxins, aflatoxin B_1, fumonisin B_1 and microcystin-LR by high content analysis

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ABSTRACT

As human co-exposure to natural toxins through food and water is inevitable, risk assessments to safeguard health are necessary. Aflatoxin B_1 and fumonisin B_1, frequent co-contaminants of maize and microcystin-LR, produced in freshwater by cyanobacteria are all naturally occurring potent toxins that threaten human health. Populations in the poorest regions of the world may suffer repeated simultaneous exposure to these contaminants.

Using High Content Analysis, multiple cytotoxicity endpoints were measured for the individual toxins and mixtures in various cell lines.

Results highlighted that significant cytotoxic effects were observed for aflatoxin B_1 in all cell lines while no cytotoxic effects were observed for fumonisin B1 or microcystin-LR. Aflatoxin B_1/microcystin-LR was cytotoxic in the order HepG2 > Caco-2 > MDBK. Fumonisin B_1/microcystin-LR affected MDBK cells. The ternary mixture was cytotoxic to all cell lines. Most combinations were additive, however antagonism was observed for binary and ternary mixtures in HepG2 and MDBK cell lines at low and high concentrations. Synergy was observed in all cell lines, including at low concentrations.

The combination of these natural toxins may pose a significant risk to populations in less developed countries. Furthermore, the study highlights the complexity around trying to regulate for human exposure to multiple contaminants.

1. Introduction

Monitoring of contaminants in water and foods has provided a huge body of evidence that humans and other organisms are exposed to complex mixtures of chemicals/natural toxins rather than to one particular compound (Altenburger et al., 2013). Current toxicological risk assessments do not adequately evaluate the impact of concomitant exposure to a number of chemical hazards (Maffini and Neltner, 2015). Traditionally these assessments have been performed for single contaminants rather than evaluating multiple compounds due to the complexities involved and the available techniques (Seidle and Stephens, 2009). So whilst the toxicity of a single contaminant on a variety of organs has been examined, simultaneous exposures to other hazards that may affect the same organs or systems in the body will not have been explored (Maffini and Neltner, 2015). As a result, vital data namely, additive, synergistic and antagonistic effects is not available for consideration by the relevant authorities (Wilson et al., 2016).

Of interest are combinations of biotoxins that occur naturally in the

Abbreviations: IARC, International Agency for Research on Cancer; HCA, High Content Analysis; HCS, High Content Screening; AFB_1, Aflatoxin B_1; FB_1, Fumonisin B_1; MC-LR, Microcystin-LR; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; MEM, minimum essential media; NEAA, non-essential amino acids solution; DMEM, Dulbecco's modified eagle medium; HepG2, human hepatocellular carcinoma cells; Caco-2, human epithelial colorectal adenocarcinoma cells; MDBK, Madin-Darby bovine kidney epithelial cells; CN, cell number; NA, nuclear area; NI, nuclear intensity; MM, mitochondrial mass; MMP, mitochondrial membrane potential; SEM, standard error of the mean; WHO, World Health Organisation; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NR, Neutral red; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide; LDH, lactate dehydrogenase; ROS, reactive oxygen species; CYP450, cytochrome P450 enzymes

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field, on crops and in freshwater lakes, rivers and ponds that may present a considerable risk to human health particularly in developing countries. These include mycotoxins, secondary metabolites of filamentous fungi that are frequent contaminants of cereal crops, and microcystins, cyanobacterial toxins that populate eutrophic freshwater bodies. One of the most important staple cereal crops in Africa, Latin America and rural China (Ranum et al., 2014; Gong et al., 2009) is maize and frequent co-contamination with aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) have been well reported in these regions (Warth et al., 2012; Kimanya et al., 2014; Adetuniji et al., 2014; Murashiki et al., 2017; Sun et al., 2011; Wang et al., 2013; Liu et al., 2016; Ono et al., 2001; Moreno et al., 2009; Torres et al., 2015; Oliveira et al., 2017). The freshwater toxin microcystin-LR (MC-LR), has also been frequently found in lakes and rivers in these regions (Sitoki et al., 2012; Mankiewicz-Boczek et al., 2015; Mbonde et al., 2015; Ueno et al., 1996; Zhang et al., 2009; Chen et al., 2009; Li et al., 2011; Vasconcelos et al., 2010; Ruiz et al., 2013; Romero-Oliva et al., 2014; Gonzalez-Piana et al., 2017) and often contaminates drinking water supplies (Codd et al., 1999; Falconer, 1999, 2005). The toxicological properties of these compounds are well documented. AFB_1 was classified as a Group 1A human carcinogen by the International Agency for Research on Cancer (IARC) in 1993 (World Health Organization International Agency for Research on Cancer, 1993), while FB_1 and MC-LR have been characterised as being possibly carcinogenic to humans (Group 2B) (World Health Organization International Agency for Research on Cancer, 1993; World Health Organization International Agency for Research on Cancer, 2010). Additionally, the World Health Organisation (WHO) introduced a drinking water guideline of 1 μg/l for MC-LR (WHO, 2003).

Given the likelihood of AFB_1, FB_1 and MC-LR co-exposure, the risks associated with this need to be elucidated to ensure that public health is protected. The aim of this study was to assess the cytotoxic and interactive effects of these three natural toxins, at realistic concentrations. These have been determined from biomarker exposure surveys for AFB_1 and FB_1 described in the literature in addition to reported MC-LR concentrations in serum from populations exposed to this biotoxin or consumption estimates (Table 1). To this end, using High Content Analysis, multiple cytotoxicity endpoints were measured for the individual toxins as well as mixtures in human hepatocellular carcinoma cells (HepG2), Madin-Darby bovine kidney epithelial cells (MDBK) and human epithelial colorectal adenocarcinoma cells (Caco-2). These cell lines replicate the organs/systems of the body that the toxins are known to affect. Cell number (CN), nuclear area (NA), nuclear intensity (NI), mitochondrial mass (MM) and mitochondrial membrane potential (MMP) were evaluated. The endpoints selected are markers of cell health and encompass the features of cellular metabolic functions (O'Brien, 2014), therefore providing valuable information for the risk assessors.

2. Materials and methods

2.1. Chemicals

Aflatoxin B_1 (AFB_1) (99.5 ± 0.5% pure) and fumonisin B_1 (FB_1) (97.6 ± 2.4% pure) were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria) and microcystin-LR (MC-LR) (≥95% pure) was provided by Enzo Life Sciences (UK) Ltd, Exeter, United Kingdom. Phosphate buffered saline (PBS), formalin solution, dimethyl sulfoxide (DMSO) (≥99.7% pure) and methanol (99% pure) were obtained from Sigma-Aldrich (Poole, UK). Minimum essential media (MEM), minimum essential media non-essential amino acids solution (NEAA), Dulbecco's modified eagle medium containing phenol red (DMEM), foetal bovine serum, L-glutamine, sodium pyruvate, penicillin streptomycin, TrypLE™ Express, valinomycin, Hoechst 33342 solution, MitoTracker® Orange CMTMRos, trypan blue and Countess™ cell counting chamber slides were purchased from Life Technologies (Paisley, Scotland). All other reagents/materials were standard laboratory grade.

2.2. Toxin mixtures

Stock solutions of AFB_1, FB_1, and MC-LR were prepared in methanol (100%) and working solutions were diluted from the stocks in cell culture medium on the day of use. The resultant methanol concentration was 0.5% (v/v). For AFB_1, the working concentrations ranged from 0.1 ng/ml to 500 ng/ml (0.32 nM–1.6 μM), for FB_1, 200 ng/ml to 8000 ng/ml (0.28 μM–11.1 μM) and for MC-LR the concentrations ranged from 0.2 ng/ml to 250 ng/ml (0.2 nM–250 nM). In addition, binary mixtures of AFB_1 and MC-LR, FB_1 and MC-LR and ternary mixtures of AFB_1, FB_1 and MC-LR were prepared in culture medium using the same concentration ranges detailed above. The experimental concentrations selected were based on estimated exposure data derived from measuring urinary biomarkers in the case of AFB_1 and FB_1 and serum concentrations or consumption data for MC-LR reported in the literature. Table 2 relates the *in vitro* concentrations used to *in vivo* concentrations in humans to enable comparison with human exposure data outlined in Table 1.

Table 1
Estimated exposure levels of populations to aflatoxins, fumonisins and microcystin-LR using biomarkers.

Country	Biomarker	Concentration (μg/L)	Estimated exposure (μg/kg/bw/d)	Reference
Cameroon	Urinary AFM_1	1.38	1.15	Abia et al., 2013
Nigeria	Urinary AFM_1	1.5	2.5	Ezekiel et al., 2014
Brazil	Urinary AFM_1	0.0069	0.0018	Jager et al., 2014
	Urinary AFM_1	0.0042	0.0014	
China – Fushui County	Urinary AFM_1	3.2	3.68	Zhu et al., 1987
China - Shanghai	Urinary AFM_1	5.2	4.33	Qian et al., 1994
Cameroon	Urinary FB_1	14.8	123.3	Abia et al., 2013
Nigeria	Urinary FB_1	12.8	76	Ezekiel et al., 2014
China – Huaian County	Urinary FB_1	13.63	7.67	Xu et al., 2010
China – Fusui County	Urinary FB_1	0.72	2.12	
Mexico	Urinary FB_1	0.147	0.368	Gong et al., 2008
South Africa	Urinary FB_1	0.225	8.14	Van der Westhuizen et al., 2011
China – Anhui Province	MC-LR (serum)	0.39	0.065	Chen et al., 2009
China- Three Gorges Reservoir	Estimated from food and water consumption	/	0.203	Li et al., 2011
Brazil, Caruaru[a]	MC-LR (serum)	133	11.1	Pouria et al., 1998

[a] Acute poisoning of dialysis patients during haemodialysis.

Table 2
Relating the *in vitro* concentrations used to *in vivo* concentrations in humans.

Toxin	Concentration (μg/L)	Concentration (nM)	Concentration in well (μg/200 μl)	Toxin (μg/kg/bw/d)[a]
Aflatoxin B_1	500	1600	100	1.6
	100	320	20	0.33
	10	32	2	0.033
	2	6.4	0.4	0.006
	0.1	0.32	0.02	0.0003
Fumonisin B_1	8000	11100	1600	26.6
	4000	5500	800	13.3
	2000	2800	400	6.67
	1000	1400	200	3.33
	200	280	40	0.67
Microcystin-LR	250	250	50	0.83
	50	50	10	0.17
	5	5	1	0.017
	1[b]	1	0.2	0.003
	0.2	0.2	0.04	0.0006

[a] Based on the average weight of an adult being 60 kg.
[b] WHO limit for MC-LR in drinking water.

2.3. Cell culture and treatment

Human hepatocellular carcinoma (HepG2) cells, human epithelial colorectal adenocarcinoma (Caco-2) cells and Madin-Darby bovine kidney epithelial (MDBK) cells were routinely cultured in 75 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) in a 5% carbon dioxide atmosphere at 37 °C and 95% relative humidity. MEM media supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine was used to maintain HepG2 and Caco-2 cell lines while MEM media containing 10% foetal bovine serum and 1% NEAA was selected for MDBK cells. TrypLE™ Express was used to dissociate the adherent cells from the flask prior to staining with trypan blue for cell counting and viability check using a Countess® automated cell counter.

Cells were seeded into Corning® BioCoat™ Collagen I, 96 well, clear flat bottom microtitre plates (Corning Life Sciences, New York, US). Seeding densities were 1×10^5 cells/ml, 8×10^4 cells/ml and 4×10^4 cells/ml for HepG2, Caco-2 and MDBK cells, respectively. These densities have been established and are standard protocol within our laboratory. They are important to ensure high quality, reproducible assay results. If the seeding densities are too low, the greater the response to the test compounds, whereas high cell numbers tend to be less responsive to the test compounds (Riss and Moravec, 2004). After 24 h, the cells were exposed to the single, binary and ternary mixtures of the test compounds described above for 48 h (five concentrations were tested for each). Negative controls of 0.5% (v/v) methanol/media and 0.1% (v/v) DMSO/media and a positive control, valinomycin prepared in DMSO (100%) and diluted in media, (final concentration of 60 μM in 0.1% (v/v) DMSO/media) were included in the study. All controls and toxin compounds/mixtures were tested in triplicate.

2.4. Cytotoxicity assessment using High Content Analysis

Two fluorescent probes were used to assess any subtle or overt changes in cell health. MitoTracker® Orange CMTMRos, enabled evaluation of mitochondrial function such as changes in mitochondrial mass (MM) or in mitochondrial membrane potential (MMP) and Hoechst nuclear stain was used to label the cell DNA, allowing measurement of features such as cell number (CN), nuclear area (NA) and nuclear intensity (NI). A stock solution of MitoTracker® Orange CMTMRos, live cell stain was prepared by adding 117 μl DMSO to 50 μg dye (1 mM solution). The stock was diluted 1:100 with DMEM media (according to the manufacturer's instructions as the dye is susceptible to potential oxidases in serum and so complete media, such as MEM, may not be used) to an intermediate concentration of 10 μM and a further 1:100 dilution in DMEM performed to give a final working solution of 100 nM. Hoechst stock solution (20 mM) was diluted to a working solution concentration of 2 μM in PBS prior to use.

Following 48 h of exposure under culture conditions, the media and test mixtures were removed carefully and the live cell stain, MitoTracker® Orange CMTMRos (50 μl) added to each well for 30 m at 37 °C, protected from the light. The mitochondrial fluorescent dye was removed and the cells were fixed with 10% formalin solution (150 μl) for 15 m at room temperature and protected from light. The fixing solution was gently removed and the cells washed with PBS (200 μl) after which the nuclear (Hoechst) stain (100 μl) was added and incubated for 20 m at room temperature and protected from the light. Finally, the cells were washed with PBS as before and fresh PBS (200 μl) added to the wells prior to sealing the plate with black vinyl film to protect the photosensitive samples until reading. Samples were evaluated on CellInsight™ NXT High Content Screening (HCS) platform (Thermo Fisher Scientific, UK). Using automated fluorescent microscopy and advance imaging tools, the instrument allows the quantitative analysis of multiple parameters for single cells (O'Brien et al., 2006). For each plate, data was captured at 10× objective magnification and five field of view images were acquired for each parameter. Excitation and emission wavelengths for Hoechst nuclear stain and MitoTracker® Orange CMTMRos were Ex/Em 361/497 nm and Ex/Em 554/576 nm, respectively.

2.5. Statistical analysis

Exposure analyses were performed in triplicate on three independent occasions and the results expressed as the mean percentage of the solvent control ± standard error of the mean (SEM) of the exposures. All data was analysed using Microsoft Excel and GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, California). Significant differences at the 95% confidence level between the control and treated groups were determined by one-way ANOVA and Dunnett's multiple comparison assessment. Significant cytotoxic injury is denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

2.6. Determination of the interactive effects of the toxins

Several experimental designs exist for the study of drug/contaminant interactions (Smith et al., 2016). The most commonly applied approach is that detailed by Weber et al. (2005) and compares theoretical expected values derived from single contaminant exposure studies with the observed values derived from co-exposure experiments. The other widely used technique is the Chou, 2006 method that uses isobologram analyses and quantification of the antagonism or synergy by calculation of a combination index (Le et al., 2018). In this study, comparison between the expected and observed measurements was performed to indicate if the interactions of the binary and ternary mixtures of toxins were additive, antagonistic or synergistic. The expected values were calculated as described by Weber et al. (2005), whereby the mean (%) of exposure to one compound was added to the mean value (%) after exposure to the second and then the third compound. To illustrate; expected values after exposure to a binary mixture were calculated as follows: mean (expected for AFB_1 + FB_1) = (mean (AFB_1) + mean (FB_1)) – 100% or in the case of exposure to a ternary mixture: mean (AFB_1 + FB_1 + MC-LR) = (mean (AFB_1 + FB_1) + mean (MC-LR)) − 100%. The expected SEM for binary combinations was calculated as follows: SEM (expected for AFB_1 + MC-LR) = $[(\text{SEM for } AFB_1)^2 + (\text{SEM MC-LR})^2]^{1/2}$ and for the ternary mixture; SEM (expected for AFB_1 + FB_1 + MC-LR) = $[(\text{SEM for } AFB_1 + FB_1)^2 + (\text{SEM for MC-LR})^2]^{1/2}$. To determine significant differences between the expected and observed measurements, an

unpaired *t*-test was applied and results considered significantly different if $p \leq 0.05$. The effects of the toxin mixtures were considered additive if the measured cell parameters were not significantly above or below the expected values. If the measured parameters were significantly greater than the expected values for the endpoints CN, NI and MMP and significantly below the expected values for NA and MM this signified antagonism. Finally, synergistic effects were indicated if the measured cell parameters were significantly less than the expected values for CN, NI and MMP and significantly greater than the expected values for the endpoints NA and MM.

3. Results

3.1. Cytotoxicity of individual toxins

The cytotoxic effects of individual naturally occurring toxins, AFB_1, FB_1 and MC-LR on three cell types (HepG2, Caco-2 and MDBK) were investigated using HCA, allowing the rapid, simultaneous interrogation of various cellular parameters such as CN, NA, NI, MM and MMP. Following 48 h of exposure, AFB_1 (at the highest concentration tested) triggered cellular injury in all cell types while no toxicity was observed for FB_1 and MC-LR at the concentrations investigated. Fig. 1 shows images of HepG2 cells following treatment with the negative and positive controls and AFB_1 at 500 ng/ml. As a result of exposure to 500 ng/ml (1.6 μM) AFB_1 in HepG2 cells, CN fell by 17.3% ($p \leq 0.001$), NA increased by 17% ($p \leq 0.001$) and MM increased by 12.3% ($p \leq 0.001$). Caco-2 cells showed increases in NA and MM of 10.1% ($p \leq 0.001$) and 8.3% ($p \leq 0.001$), respectively, and in MDBK cells, CN dropped by 13.5% ($p \leq 0.05$) while NA increased by 6.6% ($p \leq 0.01$) (Fig. 2).

3.2. Cytotoxicity of the binary mixtures AFB_1/MC-LR and FB_1/MC-LR

The cytotoxic effects of the binary toxin mixtures AFB_1/MC-LR and FB_1/MC-LR on the cellular parameters of CN, NA, NI, MM and MMP in each cell line are shown in Figs. 3 and 4, respectively. Cytotoxicity was observed for the highest concentrations of each used, that is a mixture 1.6 μM AFB_1/250 nM MC-LR, and a mixture of 11.1 μM FB_1/250 nM MC-LR. Three endpoints, CN, NA and MM were significantly altered by the binary mixture AFB_1/MC-LR in the HepG2 cells. Cell numbers dropped by 11.3% ($p \leq 0.05$) and NA and MM both showed increases of 8.2% ($p \leq 0.01$) and 10.6% ($p \leq 0.01$), respectively. This mixture also affected MM in Caco-2 cells (an increase of 5.4%, $p \leq 0.05$) and NA in MDBK cells where an increase of 3.7% was observed ($p \leq 0.01$). A decrease of 14.1% ($p \leq 0.05$) in CN and an increase of 10.2% ($p \leq 0.001$) in NI was detected in MDBK cells following treatment with FB_1/MC-LR. Mitochondrial membrane potential in all cell lines investigated remained unaltered after treatment with these binary toxin combinations.

Results of the interactive effects of AFB_1/MC-LR and FB_1/MC-LR on these particular cell lines are illustrated in Figs. 5 and 6, respectively. In HepG2 cells, antagonism was observed after 48 h treatment with all concentrations of the AFB_1/MC-LR mixture for CN. Measured values were determined to be significantly higher than those expected by 13.9% ($p \leq 0.01$), 24.6% ($p \leq 0.001$), 11.5% ($p \leq 0.05$), 12.1% ($p \leq 0.05$) and 8.2% ($p \leq 0.05$) at increasing toxins concentrations. Antagonistic effects were also evident for NA measurements using the highest concentration mixture (1.6 μM AFB_1/250 nM MC-LR) in two of the cell lines where the measured response was less than that expected by 10.4% ($p \leq 0.01$) (HepG2) and 6.1% ($p \leq 0.001$) (MDBK). For NI in HepG2 cells, synergy was observed for the mixture containing the highest concentrations of AFB_1/MC-LR.

Treatment with a binary combination of FB_1/MC-LR demonstrated antagonism between the toxins at the two lowest concentration mixtures used (0.28 μM FB_1/0.2 nM MC-LR and 1.4 μM FB_1/1 nM MC-LR) for both CN and NA in HepG2 cells. Significantly higher measured values of 12.8% ($p \leq 0.05$) and 13.6% ($p \leq 0.01$) and lower measured results of 8.3% ($p \leq 0.01$) and 7.4% ($p \leq 0.05$) compared to what was expected were observed for CN and NA, correspondingly. Antagonism was also noted for NA endpoint in MDBK cells at the highest concentration of the mixture used (11.1 μM FB_1/250 nM MC-LR), displaying a difference of 5.0% between the measured and expected values ($p \leq 0.05$). Furthermore, synergistic effects were seen at these concentrations for MMP in MDBK cells (a difference of 9.4%, $p \leq 0.05$) and for NI in Caco-2 cells where a difference of 7.1% ($p \leq 0.05$) was determined between measured and expected values. The findings for all other mixtures were considered to be additive effects because the measured values were not significantly above or below the expected results.

3.3. Cytotoxicity of the ternary combination of AFB_1/FB_1/MC-LR

The combination of all three toxins triggered a toxic response at the highest concentrations tested, namely, 500 ng/ml AFB_1, 8000 ng/ml FB_1 and 250 ng/ml MC-LR (1.6 μM, 11.1 μM and 250 nM, respectively) in various endpoint parameters for HepG2, Caco-2 and MDBK cell lines (Fig. 7). Four endpoints were significantly affected in HepG2 cells where a decrease of 13.0% ($p \leq 0.05$) was observed for CN and for NA, MM and MMP, increases of 11.3% ($p \leq 0.001$), 11.3% ($p \leq 0.01$) and 10.9% ($p \leq 0.01$), respectively, were observed. When this mixture was applied to MDBK cells, the results revealed a decrease in CN (11.5%, $p \leq 0.05$) and increases in NA (3.8%, $p \leq 0.05$), NI (6.8%, $p \leq 0.05$) and MM (5.0%, $p \leq 0.05$). The only parameter affected in the Caco-2 cell line was MM which increased by 6.8% ($p \leq 0.01$).

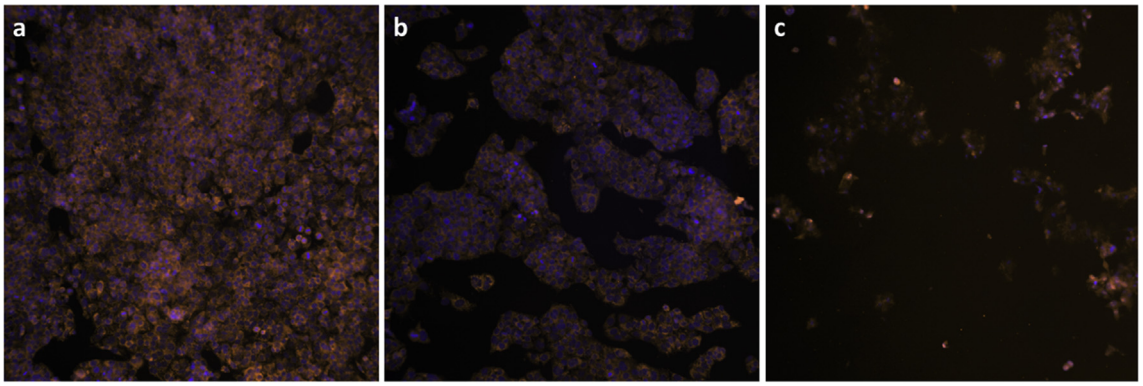

Fig. 1. HCA images for HepG2 treated cells showing (a) Negative control (0.5% (v/v) methanol), (b) AFB_1 (500 ng/ml) and (c) the positive control (60 μM valinomycin). Images were acquired at 10× objective magnification using Hoechst dye (blue, nuclear stain) and MitoTracker® Orange CMTMRos (orange, mitochondrial stain). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

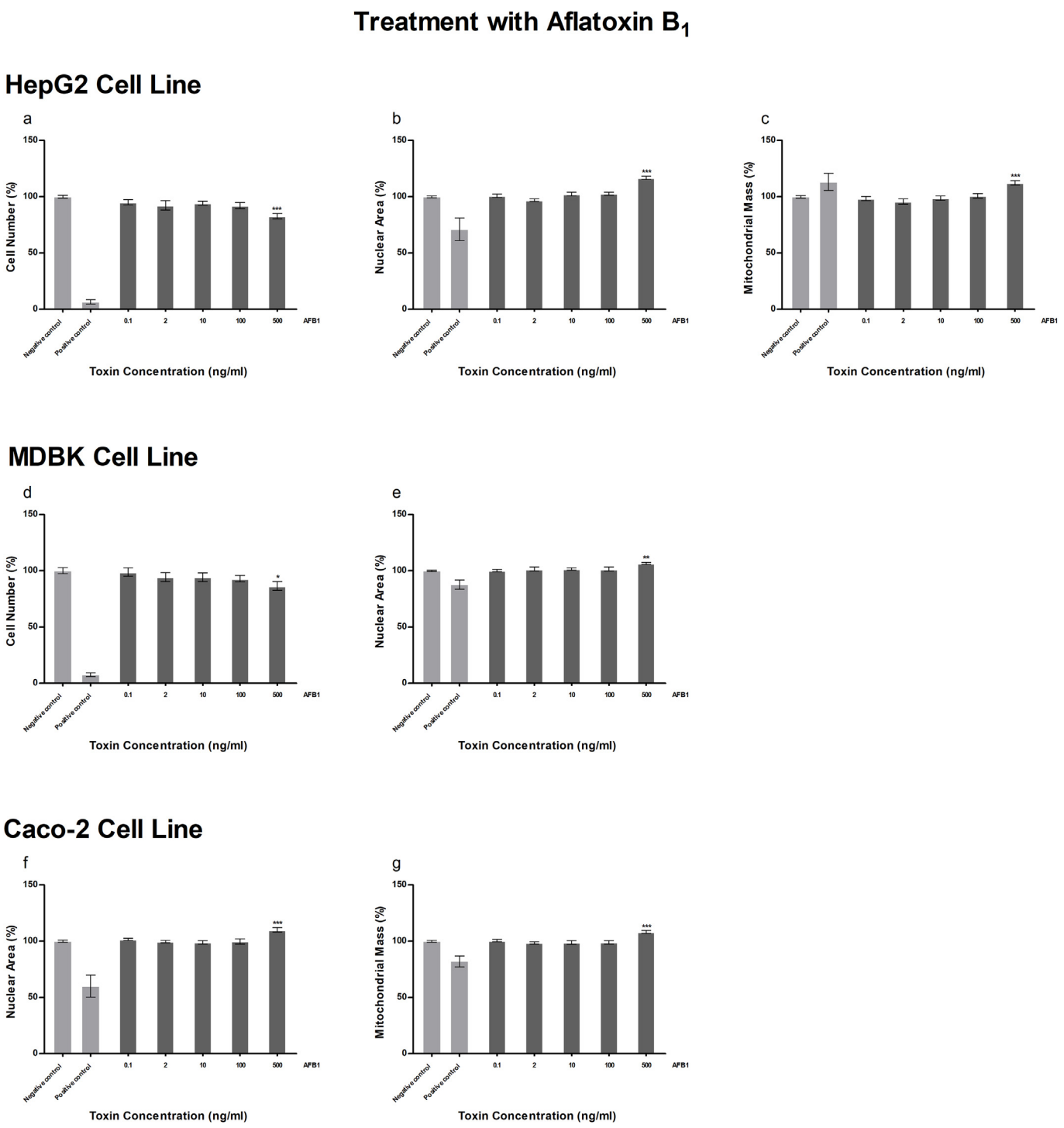

Fig. 2. Cytotoxic effects of AFB_1 after 48 h exposure on various cell lines. Parameters measured included CN, NA, NI, MM and MMP. Graphs show effects on HepG2 cells: (a) cell number (CN), (b) nuclear area (NA), (c) mitochondrial mass (MM), on MDBK cells: (d) cell number (CN) and (e) nuclear area (NA) and on Caco-2 cells: (f) nuclear area (NA) and (g) mitochondrial mass (MM). Data for each ($n = 3$) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) of the exposure parameter. Significant cytotoxicity is denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing adverse effects are shown.

The combined effects of the ternary mixtures of AFB_1, FB_1 and MC-LR were assessed and are represented in Fig. 8. The most significant outcomes were exhibited in HepG2 cells where antagonism was evident at the lower concentration ranges tested for CN and NA. Measured CN was 10.9% ($p \leq 0.05$) and 18.7% ($p \leq 0.01$) greater than the expected values while for NA, the measured result highlighted a difference of 8.4% ($p \leq 0.05$) lower than what was expected. In addition, this effect was observed for NA in MDBK cells (3% difference, $p \leq 0.01$) at the highest concentrations of toxins used. A synergistic effect was evident for a low-level concentration mixture (2 ng/ml AFB_1/1000 ng/ml FB_1/1 ng/ml MC-LR) at one endpoint (NI) in HepG2 cells where a lower measured response of 7.9% was determined ($p \leq 0.01$). As previously stated, when no significant differences were observed between the measured and expected results, the effects were considered to be additive.

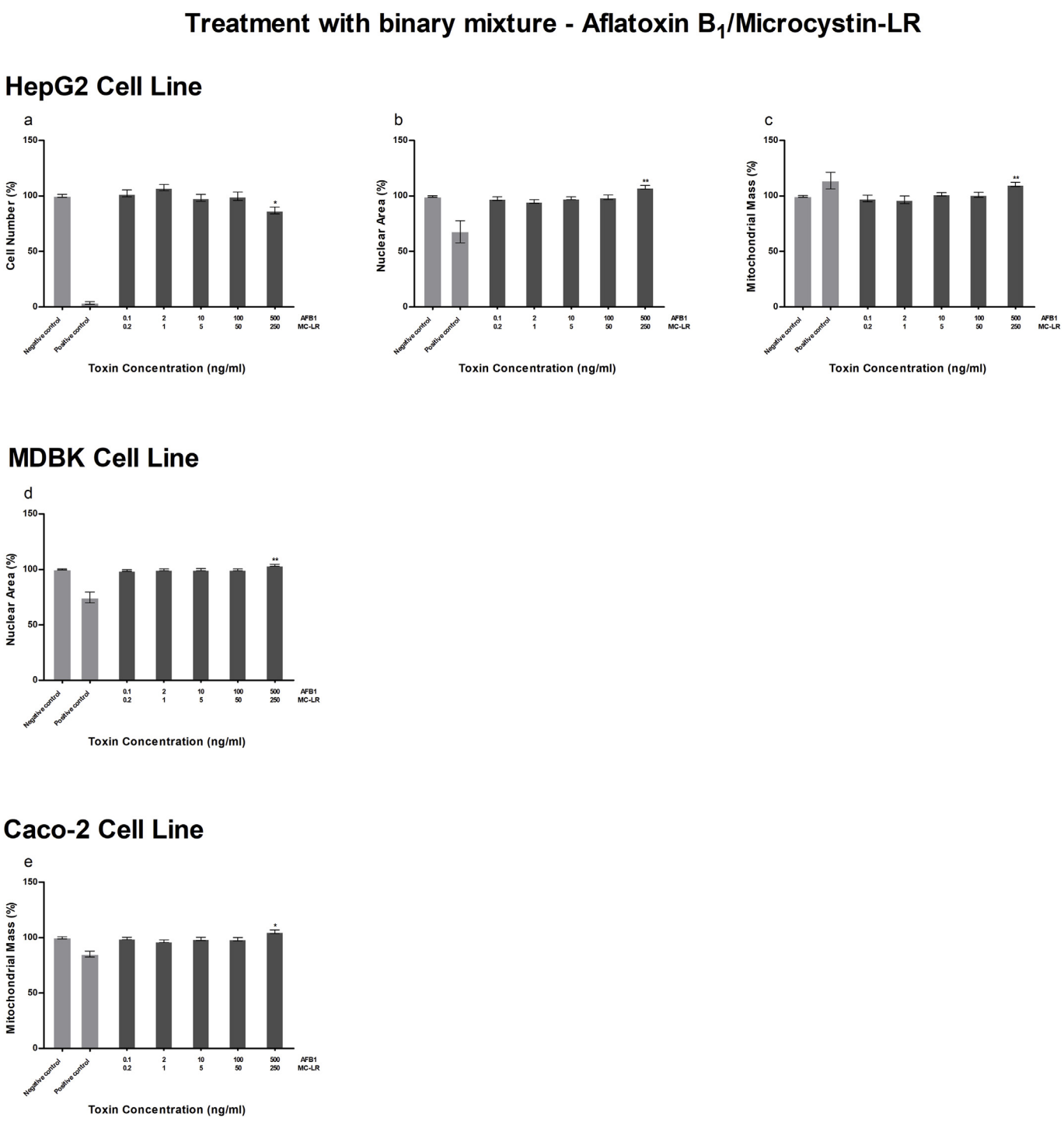

Fig. 3. Cytotoxic effects of the binary mixture AFB_1/MC-LR after 48 h exposure on various cell lines. Parameters measured included CN, NA, NI, MM and MMP. Graphs show effects on HepG2 cells: (a) cell number (CN), (b) nuclear area (NA), (c) mitochondrial mass (MM) on MDBK cells: (d) nuclear area (NA) and on Caco-2 cells: (e) mitochondrial mass (MM). Data for each ($n = 3$) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) of the exposure parameter. Significant cytotoxicity is denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing adverse effects are shown.

4. Discussion

Defining the risks of human co-exposure to multiple contaminants is a necessary but extremely complex undertaking and cytotoxicity studies are a useful initial step in determining the potential toxicities of combined test substances. In this investigation, HCA was used to reveal the toxic effects and the interactions of three naturally occurring toxins on HepG2, Caco-2 and MDBK cell lines by examining both nuclear (CN, NA, NI) and mitochondrial (MM, MMP) features. The cells were exposed to the single toxins AFB_1, FB_1 and MC-LR, in addition to binary mixtures of AFB_1/MC-LR and FB_1/MC-LR and finally ternary mixtures of all three. The concentrations chosen for the study were selected after examination of the reported exposure levels of these toxins in the applicable geographical regions (Table 1).

Cellular responses to these contaminants in various cell lines have been reported in the literature, and generally the techniques involved have been single endpoint assays such as MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), NR (Neutral Red) or WST-1,

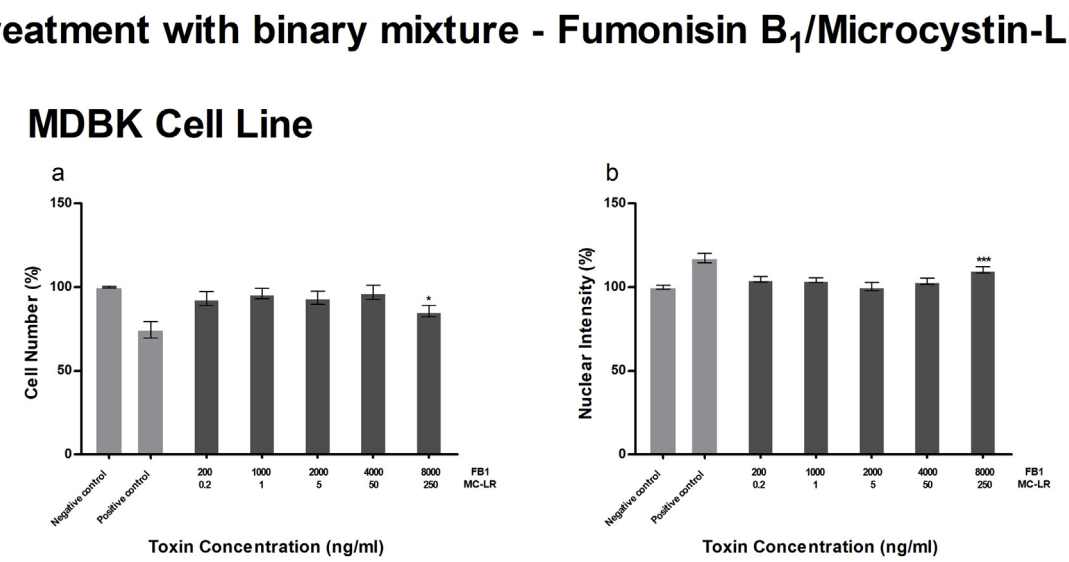

Fig. 4. Cytotoxic effects of the binary mixture FB_1/MC-LR after 48 h exposure on various cell lines. Parameters measured included CN, NA, NI, MM and MMP. Graphs show effects on MDBK cells: (a) cell number (CN) and (b) nuclear intensity (NI). Data for each (n = 3) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) of the exposure parameter. Significant cytotoxicity is denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing adverse effects are shown.

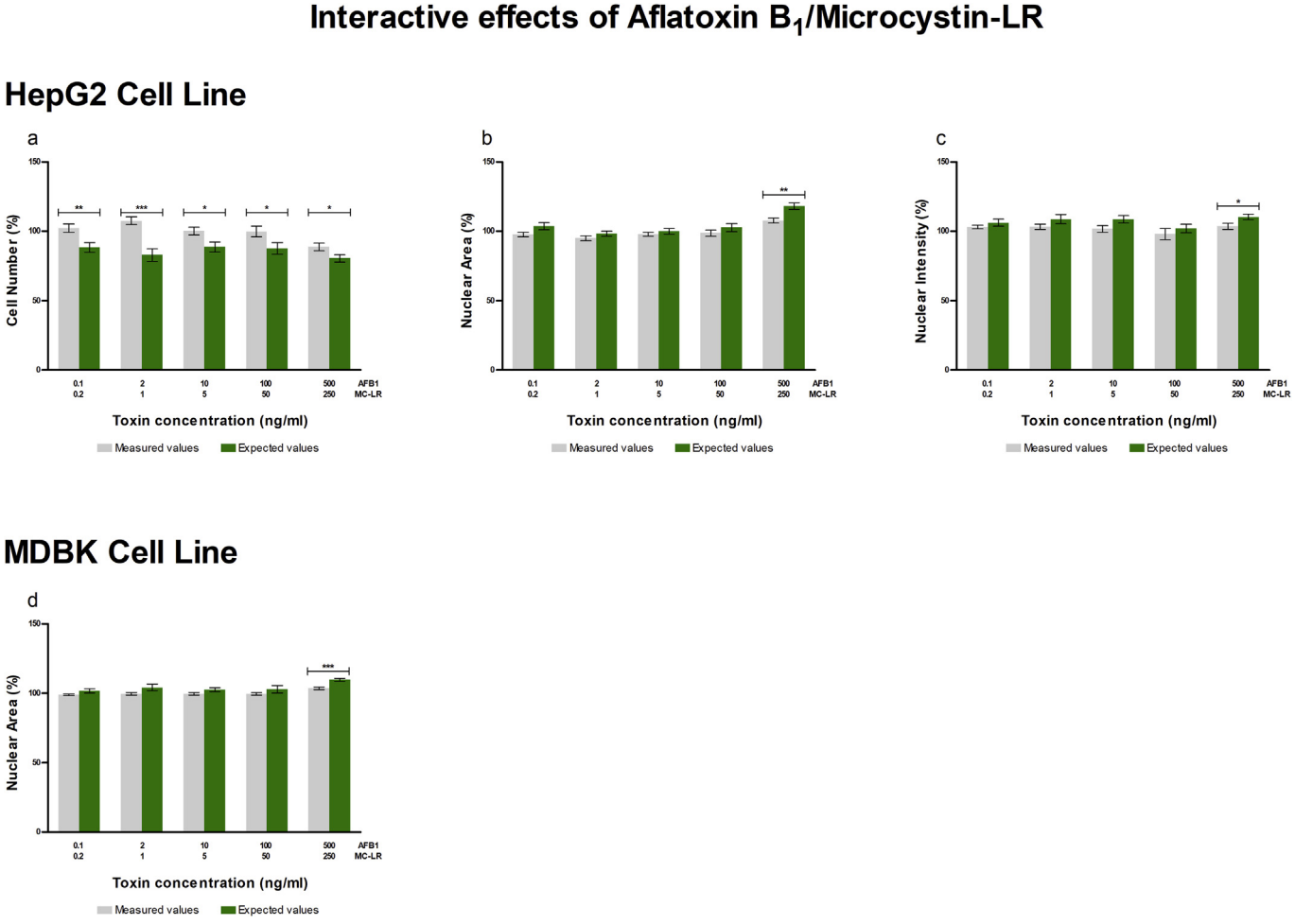

Fig. 5. Interactive cytotoxic effects of the binary mixture AFB_1/MC-LR to various endpoints after 48 h exposure to different cell lines. Effects are shown for HepG2 cells: (a) cell number CN, (b) nuclear area (NA), (c) nuclear intensity (NI), and in MDBK cells: (d) nuclear area (NA). Data for each (n = 3) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) for each parameter. Green bars denote the expected values and grey bars the measured values. Significant antagonistic and synergistic effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing effects are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

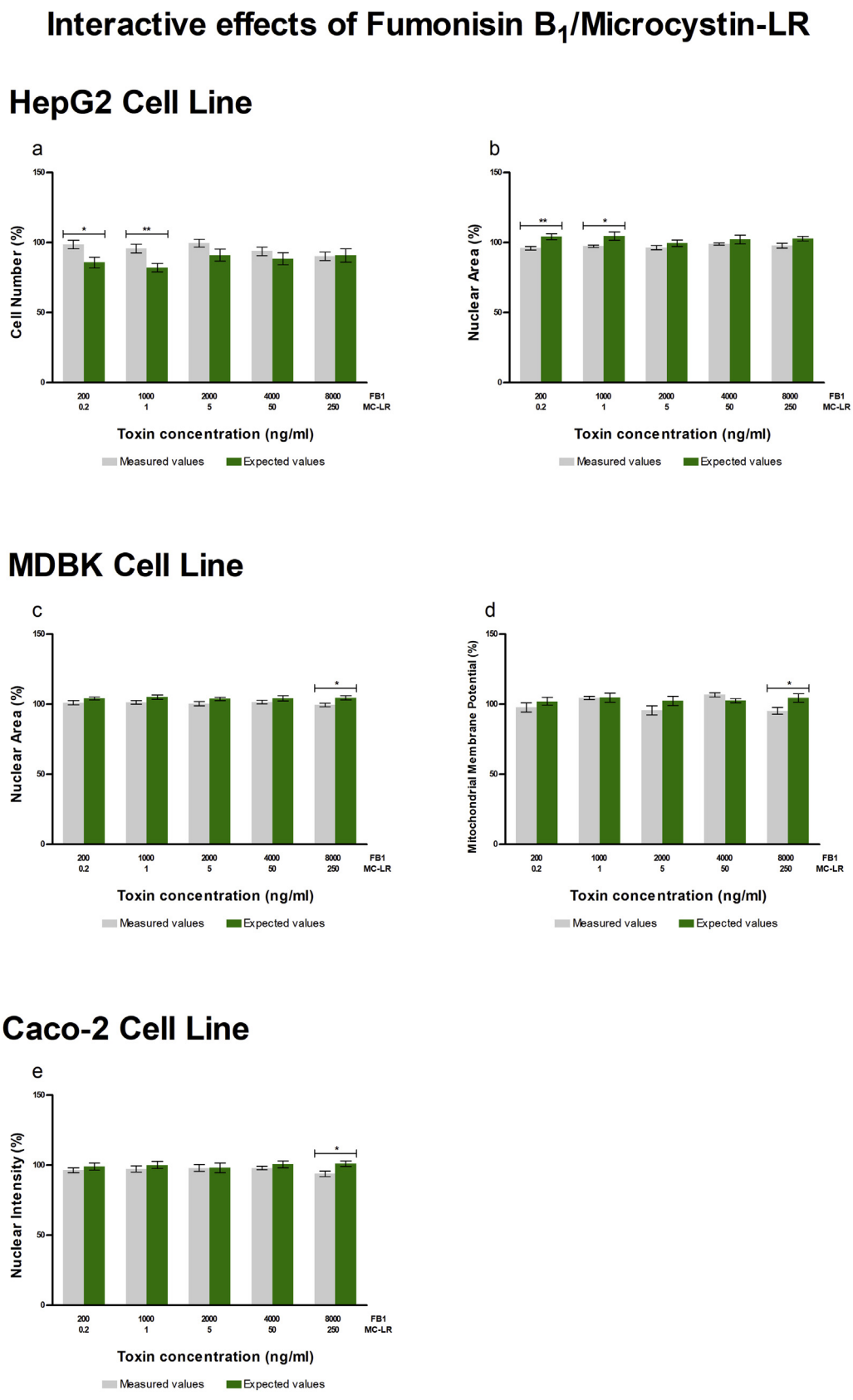

Fig. 6. Interactive cytotoxic effects of the binary mixture FB_1/MC-LR to various endpoints after 48 h exposure to different cell lines. Effects are shown for HepG2 cells: (a) cell number CN, (b) nuclear area (NA), in MDBK cells: (c) nuclear area (NA), (d) mitochondrial membrane potential (MMP) and in Caco-2 cells: (e) nuclear intensity (NI). Data for each ($n = 3$) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) for each parameter. Green bars denote the expected values and grey bars the measured values. Significant antagonistic and synergistic effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing effects are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate), amongst others. These methods tend to be less sensitive and measure lethal toxicities within a few hours, rather than allowing the treatment of cells with sub-lethal concentrations of compounds of interest for several days as with HCA (O'Brien et al., 2006). That said, there are conflicting reports in the literature regarding the comparison of CN as a measure of cell viability and the MTT assay. One group of researchers found a high concordance between the two methods

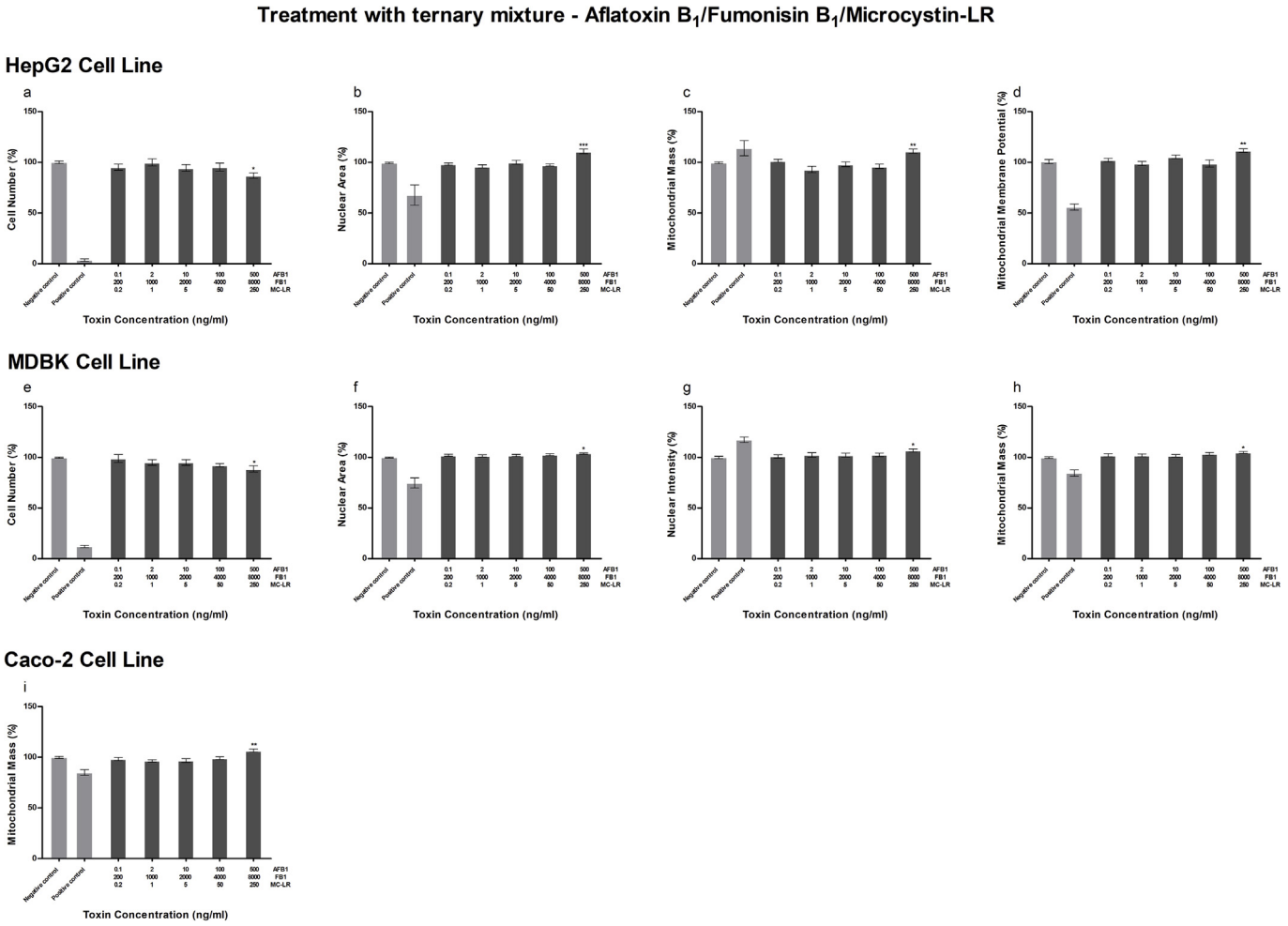

Fig. 7. Cytotoxic effects of the ternary mixture AFB_1/FB_1/MC-LR after 48 h exposure on various cell lines. Parameters measured included CN, NA, NI, MM and MMP. Graphs show effects on HepG2 cells: (a) cell number (CN), (b) nuclear area (NA), (c) mitochondrial mass (MM), (d) mitochondrial membrane potential (MMP), on MDBK cells: (e) cell number (CN), (f) nuclear area (NA), (g) nuclear intensity (NI), (h) mitochondrial mass (MM) and on Caco-2 cells: (i) mitochondrial mass (MM). Data for each ($n = 3$) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) of the exposure parameter. Significant cytotoxicity is denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Only endpoints showing adverse effects are shown.

(Wilson et al., 2016) while another group found that using HCA, CN was significantly affected, yet this was not observed in the MTT assay (Clarke et al., 2015). However, since the MTT assay measures mitochondrial activity, comparing the HCA mitochondrial parameters is imperative and in this instance it was shown by Wilson et al. (2016) that the MTT assay was less sensitive at detecting cytotoxicity. Using HCA, it has been found that in general, the marker/parameter most significantly affected by a cytotoxic agent is CN followed by NA (O'Brien, 2014) and in fact CN is still considered to be the most precise way to determine human hepatotoxicity using *in vitro* assays (O'Brien and Edvardsson, 2017). Also very sensitive to toxic compounds and affected more rapidly than other cellular parameters are mitochondria (O'Brien et al., 2006). Mitochondrial dysfunction has been associated with a wide variety of diseases in humans (Pieczenik and Neustadt, 2007), therefore analysis of MM and MMP are valuable cell health indicators. Another important nuclear morphometric feature for measuring cytotoxicity is NI (Cole et al., 2014). There are alternative sensitive cellular endpoint assays that may provide valuable information as to the effects of individual toxins and indeed on the combined toxicity of several compounds. These include measuring caspase-3 enzyme activity which is the main enzyme involved in apoptosis (Weber et al., 2005) and the measurement of reactive oxygen species (ROS), which if excessive, may stimulate cellular damage (Wilson et al., 2016).

4.1. Cytotoxicity of individual toxins

This study has shown that AFB_1 (at 1.6 μM/500 ng/ml) induced cytotoxicity in the order of HepG2 > Caco-2 > MDBK, as highlighted by the number of measured endpoints affected and how significant these changes were when compared with controls. These results concur with what can be found in the literature. It is well established that the target organ for AFB_1 is the liver and the observed findings highlight this with three endpoints exhibiting toxic responses. The mechanism of AFB_1 toxicity is well documented in the scientific literature. On entering the liver, AFB_1 undergoes metabolic activation by various cytochrome P450 (CYP450) enzymes to form the reactive toxic epoxides, exo-8,9-epoxide and endo-8,9-epoxide and other oxidation products including AFM_1, AFQ_1 and AFP_1 (Ueng et al., 1995; Kensler et al., 2011; Turner et al., 2012). Further reaction of the exo-epoxide with DNA forms an adduct with the N7 group of guanine and following depurination, aflatoxin-N7-guanine adduct is excreted in the urine along with AFM_1. Hydrolysis of the endo-epoxide results in the formation of serum adducts with proteins such as AFB_1-lysine and AFB_1-albumin. Additionally, the epoxides can react with glutathione *S*-transferases to form AFB_1-mercapturic acid which is also excreted in the urine (Kensler et al., 2011; Turner et al., 2012). The CYP450 enzymes catalysing AFB_1 epoxidation in human liver include CYP1A2 and CYP3A4 (Ueng et al., 1995) and expression of these enzymes is induced by the nuclear

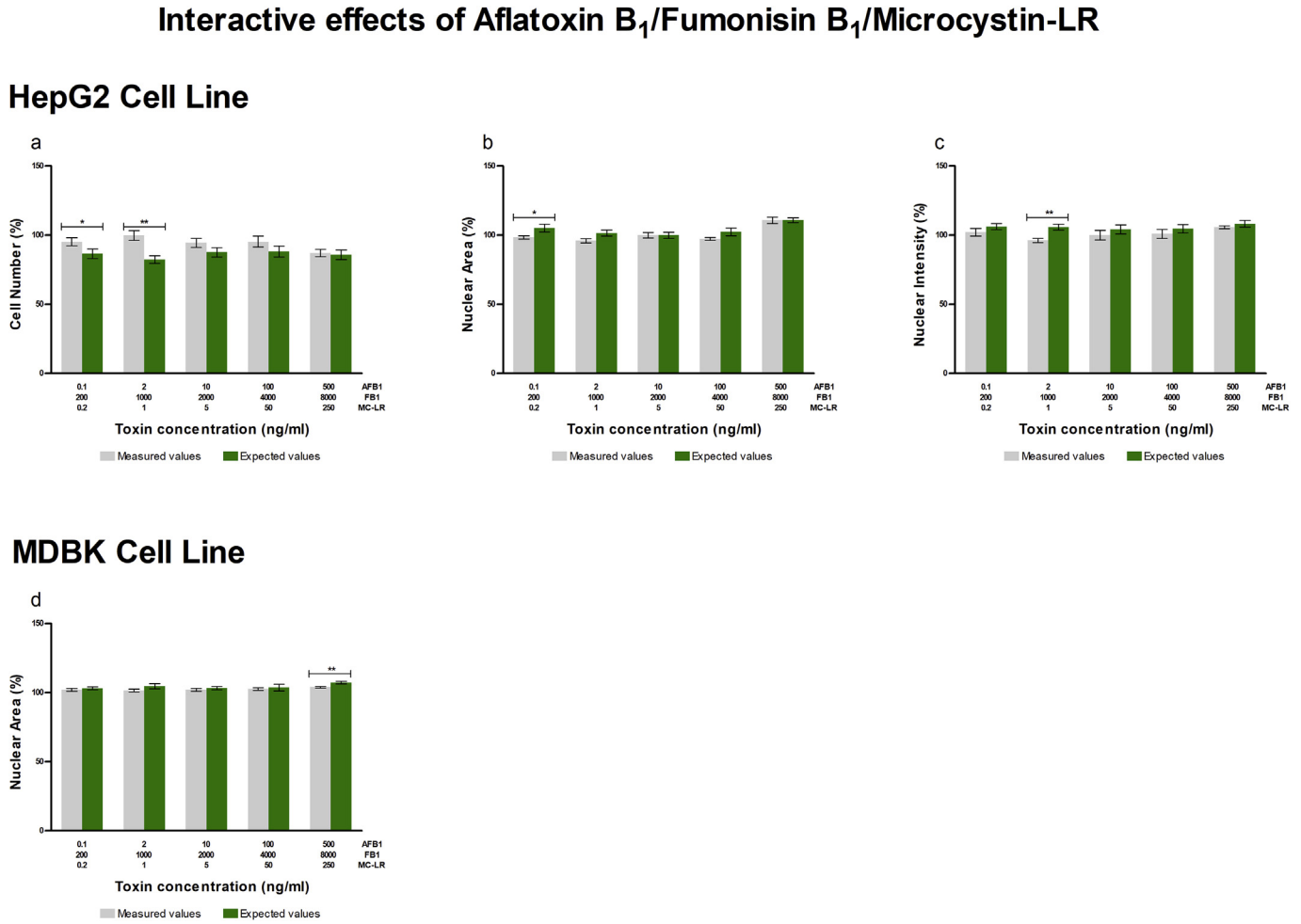

Fig. 8. Interactive cytotoxic effects of the mixture AFB_1/FB_1/MC-LR to various endpoints after 48 h exposure to different cell lines. Effects are shown for HepG2 cells (a) cell number CN, (b) nuclear area (NA), (c) nuclear intensity (NI) and in MDBK cells: (d) nuclear area (NA). Data for each ($n = 3$) is expressed as a percentage of the untreated control ± standard error of the mean (SEM) for each parameter. Green bars denote the expected values and grey bars the measured values. Significant antagonistic and synergistic effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

receptors aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Ayed-Boussema et al., 2012). These nuclear receptors are most highly expressed in the liver (Aninat et al., 2006), however have also been reported in the intestine (Tolson and Wang, 2010) and PXR has also been reported in many other tissues including kidney (Chang, 2009). Additionally, in another study comparing the transcriptomes of bovine primary cultured hepatocytes and MDBK cells, the MDBK cells had a lower abundance of many of the CYP450 enzymes required for the biotransformation of xenobiotic substances (Elgendy et al., 2017). In light of the exhibited concentrations of these nuclear receptors in the tissues outlined, this could explain the results observed in the cell lines used in this study, that AFB_1 induced cytotoxicity in the order HepG2 > Caco-2 > MDBK. Furthermore, the results of the effects in caco-2 cells agree with studies that have shown that AFB_1 impacts gut function (Grenier and Applegate, 2013; Gratz et al., 2007), that it has been shown to have serious effects on childhood nutrition (Williams et al., 2004) and is linked to growth impairment in children (Gong et al., 2002, 2003; 2004). Moreover, this biotoxin has been implicated in pathological and physiological changes in the kidney (Lin et al., 2016) and again, evidence of the cytotoxic effects of AFB_1 have been observed in this study.

Reduced cell viability, as a result of exposure to AFB_1 has been observed in a number of studies described in the literature. In 2006, McKean et al., reported that 1 μM AFB_1 caused a 50% decrease in the cell viability of HepG2 cells after 24 h of exposure when measured by WST-1 assay. Similarly, using a modified MTT assay, another research group found that the cell viability of HepG2 cells was decreased by approximately 20% following treatment with 1 μM AFB_1 (Costa et al., 2009). The results observed in this study were largely in agreement, showing a small but significant reduction in CN (17.3%, $p \leq 0.001$) of HepG2 cells following 48 h of exposure to 1.6 μM AFB_1. Additionally, significant increases (when compared to controls) were observed for both NA (17%, $p \leq 0.001$) and MM (12.3%, $p \leq 0.001$). Analysis of nuclear morphology, for example NA, may provide insights into the mechanics of cell growth and death (Boncler et al., 2017) and in fact alterations to the size and shape of the cell nucleus is associated with many types of cancer (Zink et al., 2004). Cell death attributed to necrosis is characterised by increased NA or oncosis/swelling of the cell nuclei in response to disease or injury (Vanden Berghe et al., 2014), unlike apoptosis, programmed cell death characterised by nuclear shrinkage and pyknosis (Wilson et al., 2016) in response to cell injury. In this instance, AFB_1 (1.6 μM) appears to have induced regulated necrosis (genetically controlled cell death process) as described by Vanden Berghe et al. (2014). Furthermore, increased MM, as observed, is characteristic of enhanced biogenesis as a result of increased mitochondrial respiration and is a typical response to cell damage (O'Brien and Edvardsson, 2017). Often an increase in MM is accompanied by reduced MMP (O'Brien et al., 2006), although this has not been

observed for this toxin at the concentration studied.

Similar effects were observed for Caco-2 and MDBK cells treated with AFB_1, although not to the same extent. Cell death, exemplified by a decrease in CN, was also evident for MDBK cells when treated with AFB_1 (1.6 μM); CN dropped by 13.5% ($p \leq 0.05$) when compared to the solvent control. This was similar to the findings of another study that reported a decrease in CN of 10.1% after treatment with AFB_1 (4.1 μM) when measured using the MTT assay (Clarke et al., 2014). Other research groups also reported reduced cell viability in kidney cell lines. In a porcine kidney cell line (PK-15), the IC_{50} of AFB_1 was determined to be 38.8 μM (Lei et al., 2013) while in Vero cells from green monkey kidney, the IC_{50} of AFB_1 was estimated to be 30 μM (El Golli-Bennour et al., 2010). Although there appears to be a disparity in sensitivity when comparing the kidney cell lines, these numerous experiments serve to highlight that AFB_1 induces cytotoxic effects in the kidney. As seen with the HepG2 cell line, a significant increase in NA (6.6%, $p \leq 0.01$) accompanied a drop in CN was observed in MDBK cells, thus indicating necrosis. Investigation of the effects of AFB_1 on Caco-2 cells showed increases in NA (10.1%, $p \leq 0.001$) and MM (8.3%, $p \leq 0.001$) again demonstrating cellular injury as a result of exposure to this toxin. In terms of cell viability as determined by CN, no significant decreases or increases were measured in this study. This is in total agreement with another reported study where no significant cytotoxic effects were evident using MTT and NR endpoints (Clarke et al., 2014). Furthermore, in another study (Gratz et al., 2007), a reduction of transepithelial resistance (TER) signifying cellular damage following incubation of caco-2 cells with AFB_1 (150 μM) was not associated with reduced cell viability.

No cytotoxicity, in terms of CN was exhibited in HepG2, Caco-2 or MDBK cell lines after treatment with FB_1 at concentrations of 0.28 μM–11.1 μM. This is in total agreement with many other studies investigating this mycotoxin. IC_{50} values for FB_1 in HepG2 cells have been estimated at 64.78 μM (Wentzel et al., 2017), 200 μM (Chuturgoon et al., 2015) and 399.2 μM (McKean et al., 2006). Additionally, in primary rat hepatocytes treated with 50 μM FB_1 (Ribeiro et al., 2010) or 200 μM FB_1 (Sun et al., 2015), no negative impact was observed on cell viability. Examination of the effect of FB_1 on Caco-2 cells has also shown that this cell line did not exhibit signs of toxicity at concentrations ranging from 1.4 μM to 138 μM (Caloni et al., 2002), at 13.9 μM (Clarke et al., 2014), or 0.1 μM–10 μM (Fernández-Blanco et al., 2016). No adverse effects were displayed when FB_1 was incubated with MDBK cells, also in agreement with the study by Clarke et al. (2014).

The cyanotoxin, MC-LR was also tested in HepG2, Caco-2 and MDBK cell lines and, as seen for FB_1, no effects were observed in any of the cell lines at concentrations ranging from 0.2 nM to 250 nM. The results for HepG2 cells are in complete accordance with what has been published in the literature. McDermott et al. (1998) demonstrated that in primary rat hepatocytes treated with concentrations ranging from 0.01 μM to 2 μM MC-LR, no significant differences in viability were found when compared with the untreated control cells. These results were further substantiated in various other studies in which HepG2 cells were exposed to concentrations of 10 nM, 100 nM and 1 μM (Žegura et al., 2003), at concentrations of 1 nM to 1 μM (Ikehara et al., 2015) and MC-LR concentrations of 0.1 nM–10 μM (Ma et al., 2017). Moreover, in the presence of 1 μM MC-LR, no morphological changes were induced (Ikehara et al., 2015). In complete contrast to these findings in an experiment using primary mouse hepatocytes, MC-LR at concentrations of 1 nM and 3 nM were found to induce increases in cell numbers up to 42 h after exposure, with 1 nM producing significant increases (Humpage and Falconer, 1999). Additionally, this research group revealed that, treatment with 10 nM MC-LR was found to decrease cell numbers by 50% after 18 h which indicates that mouse liver cells may be more sensitive to the effects of this toxin. Furthermore, the effects of MC-LR were compared in HepG2 cells and in normal human hepatocytes (h-Nheps) and the results highlighted that while no morphological or viability changes were found at concentrations of 1 nM–1000 nM in HepG2 cells, nuclear morphology was altered and cellular viability was inhibited in the h-Nheps cell line (Ikehara et al., 2015).

In relation to the impact of MC-LR on Caco-2 cells, the research is contradictory. Our findings correspond with the work of Vesterkvist et al. (2012). This research group tested MC-LR at concentrations of 1 μM, 50 μM and 100 μM and no significant alterations were evident using the WST-1 assay for cell proliferation or the lactate dehydrogenase assay (LDH) for quantification of plasma membrane damage. Contrary to this, cytotoxicity was found following exposure to 50 μM and 100 μM MC-LR as determined by the NR assay and total protein content, although little difference was observed at these concentrations using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-S-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide) assay (Huguet et al., 2013). Another group also noted that after 48 h of exposure, the IC_{50} value for MC-LR (total protein content) was 115.7 ± 9.2 μM and for NR uptake, it was more pronounced at 139.8 ± 11.2 μM. The morphological effects when examined by light microscopy showed, not only cellular reduction but also cell shrinkage at 200 μM (Puerto et al., 2010). While our findings are not in agreement with the two latter experiments described, this may be attributed to the concentration ranges investigated.

At the concentrations tested, 0.2 nM–250 nM, MC-LR did not induce any cytotoxic effects in MDBK cells. Once again, these findings conflict with another study reported in the literature where cell viability in human embryonic kidney and human kidney adenocarcinoma cell lines significantly decreased after treatment with 50 μM MC-LR (Piyathilaka et al., 2015). As outlined previously, the concentrations tested in both studies differ by a factor of at least 200-fold and thus in all likelihood, account for the difference. Furthermore, choice of cell line will have a huge impact due to different sensitivities of cell lines to the toxins as observed for HepG2, h-Nheps and primary mouse hepatocytes.

4.2. Cytotoxicity of the binary mixtures AFB_1/MC-LR and FB_1/MC-LR

The binary mixture of AFB_1/MC-LR appeared to induce more cytotoxic effects when compared to FB_1/MC-LR and alterations in the cells were only observed for the highest concentrations tested for each mixture. In terms of cellular damage induced by AFB_1/MC-LR, the results were in the order of HepG2 > Caco-2 > MDBK. Three endpoints were affected in the HepG2 cell line, namely, CN, NA and MM. Although significant, the decrease and increases in CN, NA and MM, respectively, were slightly less than those found for AFB_1 alone. A similar trend was observed for MM and NA in Caco-2 and MDBK cells. Antagonism was displayed for CN with this combination of toxins at all concentrations tested in HepG2 cells in addition to NA in both HepG2 and MDBK cells. These results were surprising and while there are no other studies of these mixtures, to our knowledge to compare with, some of the literature detailing the toxic effects of MC-LR may help to explain our findings. Many of the studies, as detailed previously, indicate that no toxic effects were seen (using higher concentrations) in HepG2 cells, however use of alternative cell lines did provide contrasting results (Ikehara et al., 2015; Humpage and Falconer, 1999) and if fact using sub-lethal concentrations of 1 nM–3 nM (the same range used in this study), MC-LR caused cell proliferation in primary mouse hepatocytes (Humpage and Falconer, 1999). NI is an important nuclear parameter in assessing cellular injury or damage and in the event of increasing cytotoxicity, the nucleus will swell and the NI will decrease (Cole et al., 2014). The results of this study highlighted that synergy was displayed for the highest concentration of AFB_1/MC-LR in NI of the HepG2 cells. Several retrospective epidemiological studies in China have suggested that simultaneous exposure to MC-LR and AFB_1 through drinking water and food may exacerbate the high incidences of primary liver cancer in China, (Ueno et al., 1996; Liu et al., 2017), therefore the assumption would be to expect synergistic effects in many of the endpoints measured. However, these were not the findings of this study stressing the complexity of interpretation of results and of the need for further assessments. That said, the results are also expected as dual

phase reactions are commonplace when investigating complex mixture effects (McKean et al., 2006).

The combination of FB_1/MC-LR induced cytotoxicity only in the MDBK cell line where a significant decrease and increase was measured in CN and NI, respectively, indicating cell death by apoptosis. The concentrations triggering this damage were 11.1 μM FB_1/250 nM MC-LR and although, to our knowledge, studies of this combination have not been reported, both FB_1 and MC-LR individually have been shown to induce apoptosis (Humpage and Falconer, 1999; Ribeiro et al., 2010). In HepG2 cells at concentrations of 0.28 μM FB_1/0.2 nM MC-LR and 1.4 μM FB_1/1 nM MC-LR, i.e. at and below WHO limits for MC-LR in water and at the highest mixture concentration (11.1 μM FB_1/250 nM MC-LR) in MDBK cells, antagonism was displayed. In contrast, synergistic effects were observed for the endpoints NI and MMP in Caco-2 and MDBK cell lines, respectively, at the highest concentrations tested.

4.3. Cytotoxicity of the ternary combination of AFB_1/FB_1/MC-LR

Only the ternary combination containing the highest concentration of each toxin (1.6 μM AFB_1/11.1 μM FB_1/250 nM MC-LR) caused cytotoxic effects on the cell lines. HepG2 cells were found to be the most sensitive to the ternary mixtures tested, followed by MDBK cells and finally Caco-2 cells. CN, NA, MM and MMP were all significantly affected in HepG2 cells. Decreased CN, and increased NA and MM, as discussed previously suggest that the cell death was as a result of necrosis, however, with an increase in MMP, that suggests the apoptotic pathway. This may be explained by the "apoptosis-necrosis continuum" as described by Zeiss (2003), whereby necrosis or apoptosis are triggered through a shared biochemical cascade and cell death may be expressed as one or the other depending on the physiological conditions. Similarly, this mechanism was observed for MDBK cells with typical trends for necrosis observed for CN, NA and MM, however more indicative of apoptosis was an increase in NI. Increased MM, was the only parameter affected in Caco-2 cells. In terms of the interactive effects of the toxins, the most significant results were observed in the HepG2 cell line. At the two lowest concentrations tested, antagonism was evident for the endpoints of CN and NA, an outcome that was seen when examining the results of the binary mixtures in this cell line. Antagonism was also observed at the highest concentrations of these three toxins in the NA endpoint for MDBK cells. Of note, synergy was revealed for NI at the lower end of the concentration range (6.4 nM AFB_1/1.4 μM FB_1/1 nM MC-LR).

5. Conclusion

The present study was designed to investigate the combined effects of AFB_1, FB_1 and MC-LR at realistic exposure concentrations pertinent to the poorest populations living in underdeveloped countries such as Africa, Latin America and East and South Asia. The results have confirmed that, at the exposure levels reported for these countries, single, binary and ternary combinations of these toxins may pose a considerable risk to human health. The cytotoxicity revealed for AFB_1, AFB_1/MC-LR, FB_1/MC-LR and AFB_1/FB_1/MC-LR at the highest concentrations tested indicate there is clearly a real threat to populations in East and South Asia and Africa, as, in some instances, they have been found to be exposed to higher levels of these natural toxins, (Table 1). For the majority of the parameters tested using the specified binary and ternary mixtures, additive responses were observed, while antagonism was determined for some of the mixtures. More importantly however, synergistic effects were observed for some endpoints at the highest concentrations tested in this study (AFB_1/MC-LR and FB_1/MC-LR). Even at low concentrations synergy was exhibited for the ternary mixture (AFB_1/FB_1/MC-LR) equivalent to exposures of 0.006, 3.33 and 0.003 μg/kg/bw/d for AFB_1, FB_1 and MC-LR, respectively. It is clear that populations can be exposed to these biotoxins at these levels (Table 1), therefore they present a significant potential threat. Moreover, the study has revealed that further research is required using different cell lines and using additional *in vitro* parameters to understand the mechanics of these toxicological interactions in order to protect public health.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.09.052.

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