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Combined Low-Dose Zearalenone and Aflatoxin B1 on Cell Growth and Cell-Cycle Progression in Breast Cancer MCF-7 Cells

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Abstract

Zearalenone (ZEA) has long been recognized as a xenoestrogen, while the endocrine disrupting effects of aflatoxin B1 (AFB1) have been identified recently. Due to co-occurrence and endocrine disrupting potentials of ZEA and AFB1, it was hypothesized that co-exposure to ZEA and AFB1 might affect breast cancer cell growth. Consequently, the aim of this study was to evaluate the combined effects of ZEA and AFB1 (1 nM to 100 nM) on cell growth and cell cycle progression, using a human breast cancer cell line MCF-7. Our results showed that ZEA and AFB1 produced significant interactive effects on cell growth, DNA synthesis and cell cycle progression. While ZEA promoted growth, DNA synthesis and cell cycle progression, AFB1 was cytotoxic and counteracted the effects of ZEA. ZEA altered the expression of several breast cancer related genes, whereas AFB1 had minimal effects on gene expression. With the use of specific inhibitors, ER α , GPER and MAPK pathways were found to be responsible for ZEA's effects on cell growth; while MAPK pathways might be involved in cytotoxic effects by AFB1. This study is first to report the effects of co-exposure of ZEA and AFB1 on breast cancer cell growth, possibly through ER dependent pathway. This suggested that endocrine-disrupting mycotoxins that co-occur in human food can interact and influence human health. Future work on interactive effects of endocrine-disrupting mycotoxins or other xenoestrogens is warranted, which will contribute to improved risk assessments.

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Keywords

Aflatoxin B1; Breast cancer; Endocrine disruptor; Mixture; Proliferation; Zearalenone

1. Introduction

Breast cancer is of great public concern as it has the highest incidence and mortality rates among all the woman cancers worldwide (Ferlay et al., 2013). Major risk factors of breast cancer include genetic predisposition (Dunning et al., 1999), reproductive history (Shantakumar et al., 2007) and lifetime exposure of breast tissue to estrogens (Paffenbarger et al., 1980; Pike et al., 1993; Rosner and Colditz, 1996). However, the breast cancer incidence rates are continuously increasing in both developed and developing countries and this trend could not be explained by the risk factors stated above (Ferlay et al., 2013). Recent research started to propose the involvement of environmental endocrine disruptors, especially those interfering with estrogenic pathways, as one of the causes of increasing incidence rates of breast cancer (World Health Organization and United Nations Environment Programme (WHO/UNEP), 2013).

Mycotoxins zearalenone (ZEA) and aflatoxin B1 (AFB1) are secondary metabolites produced by a few fungal species of *Fusarium* and *Aspergillus*, respectively. They can be found in cereal crops particularly those in developing countries as the food is constantly stored and processed in poor conditions (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2000, 2001). ZEA has long been recognized as a non-steroidal estrogenic compound that causes hyperestrogenism and anabolic activity in reproductive organs of animals (Kuiper-Goodman et al., 1987). It can bind to and activate the estrogen receptors (ERs) with an efficacy similar to 17 β -estradiol (E₂) but with higher potency on ER α than on ER β (Mueller et al., 2004), which implies ZEA's growth promoting effect in breast tissues. This growth promoting effect is further supported by its stimulating effect in breast cancer MCF-7 cells (Dees et al., 1997; Makela et al., 1994) and its capability to induce spontaneous tumors in mice (Schoental, 1974).

Unlike ZEA as a well-known estrogenic compound, AFB1 is commonly known as a risk factor of primary hepatocellular carcinoma (HCC; Hamid et al., 2013), and its endocrine disrupting properties has just been explored recently. Storvik et al. (2011) was the first to suggest that AFB1 might be a potential endocrine disruptor by up-regulating CYP19A1 expression in human placental JEG-3 cells *in vitro*. The mRNA expression of other enzymes responsible for steroid hormone synthesis and conjugation were also found to be increased after JEG-3 cells were exposed to AFB1 (Huuskonen et al., 2013).

There is convincing epidemiological evidence showing exposure to endocrine disrupting chemicals (EDCs) such as polychlorinated biphenyls (PCBs) (Brody et al., 2007) and diethylstilbestrol (DES) (Hilakivi-Clarke, 2014) is linked to increased breast cancer risks. EDCs acting through different pathways can act together with endogenous estrogens to provide combinatorial effects and raise the total estrogenic burden (Kortenkamp, 2007). Due to the fact that both ZEA and AFB1 are endocrine disruptors interrupting the estrogenic pathway, there might be a connection between exposure to these mycotoxins and breast

cancer. Although effects of ZEA in breast cancer have been studied for a period of time, studies of combined effect of ZEA with other mycotoxins was lacking. There are a number of studies showing that ZEA and AFB1 coexist in food and feed (Abdallah et al., 2017; Alim et al., 2018; Almeida et al., 2013; Iqbal et al., 2016; Li et al., 2013) and our laboratory also showed that AFB1 and ZEA were present in the sera and urine of a population of Egyptian women (Piekkola et al., 2012).

Consequently, our hypothesis was that ZEA and AFB1 may perturb the growth and cell cycle progression of breast cancer cells. In order to address this hypothesis, hormonal-dependent breast cancer cell line MCF-7 was used as an *in vitro* model. The doses tested were 0.01 nM to 100 nM equivalent to about 0.003 to 30 ng/mL for ZEA and AFB1. This dose range is comparable to the Provisional Maximum Tolerable Daily Intake (PMTDI) of ZEA (0.5 µg/kg body weight, corresponding to 7 ng/mL for a 70 kg man; JECFA 2000) and AFB1 level found in urine samples from the Philippines (4.25 ng/mL; Wild et al., 1986). The ultimate aim of the study was to evaluate the combined effects of ZEA and AFB1 on (i) breast cancer cell growth, (ii) the underlying direct ER dependent mechanisms through the activation of ERs and rapid cell signaling

2. Materials and Methods

2.1 Cell Culture.

Human breast cancer cell line MCF-7 were maintained in phenol red-free Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37 °C, in a humidified atmosphere containing 5% CO₂. Cells were routinely tested using MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) and were found to be free of mycoplasma contamination. Three days before treatment, the cells were incubated with DMEM/F12 supplemented with 2% charcoal-stripped FBS (Sigma-Aldrich, St. Louis, MO, USA). Aflatoxin B1 (AFB1), zearalenone (ZEA) and 17βestradiol (E₂) were purchased from Sigma-Aldrich. The compounds were dissolved in ethanol at 0.01 M and stored at -20 °C before use. The concentrations were confirmed with UV spectrophotometry. Dosing solutions were prepared by diluting the chemical stock with fresh dosing media to the desired concentrations. The final concentration of ethanol was 0.1% (v/v) in the medium, which had no effect on the cells. Negative control wells were dosed with media plus 0.1% (v/v) ethanol. Positive control wells were dosed with 1 nM E₂ (Sigma-Aldrich, St. Louis, MO, USA)

2.2 Cell Viability Assay.

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 4000 cells/well in 96-well plates (Corning, NY, USA) and were exposed to ZEA (10⁻¹² to 10⁻⁵ M) and/or AFB1 (10⁻¹² to 10⁻⁶ M) for 5 days. At the end of treatments, 10 µL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) (5 mg/mL in phosphate-buffered saline; PBS; Thermo Fisher Scientific, Waltham, MA, USA) was added to each well, and 2 hours later the media was discarded. 100 µL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each

well and the plate was shaken for 5 minutes to solubilize the purple formazan crystals formed.

Absorbance was measured at 570 nm using an iMark™ Microplate Reader (Bio-Rad Laboratories, Richmond, CA, USA). The viability of MCF-7 cells after treatment with mycotoxins was expressed as the proportion of optical density (OD) compared to control (untreated cells). The experiments were repeated four times with six replicates for each treatment.

2.3 Cell Proliferation Assay.

Cells were treated as described for MTT assay and for the last 24 hours 5-bromo-2'-deoxyuridine (BrdU) was added to culture medium. Incorporated BrdU was detected using the BrdU cell proliferation assay kit according to the manufacturer's instructions (Millipore; Billerica, MA, USA). BrdU incorporation of each sample was calculated as OD of the treatment sample minus the OD mean of control without addition of BrdU. The experiments were repeated four times with three replicates for each treatment.

2.4 Cell Cycle Analysis.

The effects of different treatments on cell cycle progression were analyzed by flow cytometry. Briefly, MCF-7 cells were plated at 1×10^6 cells in each 60 mm dish. The cells were treated with the indicated concentrations of ZEA and AFB1 (see figure legends) for 24 hours. The cells were trypsinized, washed with PBS, fixed with 70% ethanol on ice followed by resuspension in 500 μ L of propidium iodide solution (PBS containing 10 mM Tris base, 10 mM NaCl, 700 U/L RNase A, 50.1 mg/L PI and 0.1% (v/v) Nonidet P-40) to stain the cells. The cell suspensions were assayed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at the excitation wavelength 488 nm and emission wavelength 620 nm. The 10,000 gated events were collected by CellQuest Pro software (BD Biosciences), and flow cytometry data were analyzed by WinList and ModFit from Verity Software House (Topsham, ME, USA).

2.5 Gene Expression Profiling.

A commercially available Human Breast Cancer RT² Profiler™ PCR Array (Cat. No. PAHS-131ZC; Qiagen; Limburg, Netherlands) was employed to study the expression profiles of 84 genes that were commonly involved in breast carcinogenesis. Cells were seeded at 4×10^5 cells/ well in 6-well plates. The cells were treated with the indicated concentrations of ZEA and AFB1 (see figure legends) for 24 hours. RNA was isolated using Illustra RNAspin Mini RNA Isolation Kit (GE healthcare, Buckinghamshire, UK). The single strand cDNA from 1 μ g total RNA was synthesized using RT² First Strand Kit (SABioscience). Real-time polymerase chain reaction (PCR) was performed according to the user manual of RT² Profiler PCR array system (SABioscience) using RT² SYBR® Green qPCR Mastermix in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the data were analyzed using the comparative CT method by the web-based software RT² Profiler PCR Array Data Analysis version 3.5 provided by Qiagen, available at <http://www.sabiosciences.com/pcrarraydataanalysis.php>. The mRNA expression levels of target genes were normalized to β -actin (ACTB), β -2-microglobulin (B2M) and

large ribosomal protein P0 (RPLP0) as housekeeping genes. Average-linkage hierarchical clustering was generated by the RT² Profiler PCR Array Data Analysis. Significant differential gene expression (≥ 2 folds) between mycotoxins treated cells and untreated cells were identified and considered. Final filtered gene lists generated were input into R software v.3.3.2 (<https://www.r-project.org/>) (R Core Team, 2016) in combination with RStudio v. 1.0.136 (RStudio, Inc., Boston, MA, USA) to create Venn diagrams. Non-parametric Spearman correlations of gene expression profiles were analyzed by GraphPad Prism 6.0 (Graphpad Software Inc., San Diego, CA, USA). The reliability of the expression array data was confirmed by performing quantitative real-time PCR analysis (qPCR).

2.6 qPCR.

Other than PCR Array, qPCR was performed to quantify mRNA expression levels for genes encoding estrogen receptors (i.e. estrogen receptor alpha (ERα), G protein-coupled estrogen receptor 1 (GPER)) relative to RPLP0. RPLP0 was used as an internal reference control as its expression is not affected by exposure to estrogen (Laborda, 1991). RNA isolation and cDNA synthesis were performed as described before (Ling et al., 2016). qPCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems) using 1 μL of cDNA and SYBR Premix Ex Taq II Master Mix (Takara, Dalin, China), with final primer concentrations of 0.5 μM per primer in a final volume of 10 μL. Human nucleotide sequences of the primers (Supplementary Table 1) were generated from published GenBank sequences using Primer Premier 6.0 software (PREMIER Biosoft, Palo Alto, CA, USA). Samples were thermocycled using the default fast program (40 cycles of 95 °C for 5 s and 60 °C for 30 s). The experiments were repeated four times with three replicates for each treatment. All PCR reactions were performed in triplicate. Relative changes in gene expression levels in cultured breast cancer cells were analyzed using the 2^{-CT} method as described previously (Livak and Schmittgen, 2001).

Inhibition Studies.—To examine the involvement of ERα, GPER and different mitogen-activated protein kinase (MAPK) signaling pathways in affecting the viability of MCF-7 cells exposed to ZEA and AFB1, specific inhibitors of ERs and MAPKs were used. MCF-7 cells (4 × 10⁵ cells/well) were seeded overnight in 6-well plates, and then exposed to mycotoxins with and without the addition of ERα inhibitor MPP dihydrochloride (5 μM; Tocris Bioscience, Bristol, UK), GPER inhibitor G-15 (5 μM; Tocris Bioscience), MAPK kinase inhibitor PD98059 (0.2 μM; Merck Millipore, MA, USA), p38 inhibitor SB203580 (0.1 μM; Merck Millipore), and INK inhibitor II (0.2 μM; Merck Millipore). Treatments of cells with 0.1% ethanol and 1 nM E₂ were included as untreated and positive controls, respectively. MTT assay was performed after the 5day incubation of ZEA and/or AFB1 as described above. The experiments were repeated four times with six replicates for each treatment.

2.7 Statistical analyses.

Results were expressed as mean ± 95% confidence interval of four individual experiments with triplicates unless otherwise stated. Log-transformation was conducted when necessary. Statistical analyses were carried out with GraphPad Prism 6.0 (Graphpad Software Inc.). Data were first evaluated for normality with the Shapiro–Wilk and Levene's variance

homogeneity test. One-way or two-way analysis of variance (ANOVA) were conducted followed by Tukey's post hoc test for multiple group comparisons. 4-parameter nonlinear regression was used for dose-response curve fitting. Differences between dose-response curves and best-fit values were evaluated by Extra-sum-of-squares F test. Significant differences between untreated and positive controls were analyzed by two-sample t-test with unequal variances. P value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Combined Effects of ZEA and AFB1 on MCF-7 Cell Proliferation and Cell Cycle Progression.

The viability of MCF-7 cells after exposure to various concentrations of ZEA and AFB1 individually was first determined by MTT assay. Preliminary experiments have shown that 5-day exposure is optimal for E₂ and ZEA to show significant differences compared with negative control. The one-way ANOVA results showed that there were significant differences between effects produced by different concentrations of individual mycotoxins ($F_{8, 27} = 107.4$, $p < 0.001$ for ZEA and $F_{7, 24} = 131.1$, $p < 0.001$ for AFB1). Significant increase in viability by ZEA was detected from 10^{-9} up to 10^{-6} M (Figure 1A). For AFB1, significant decrease in viability was seen above 10^{-7} M (Figure 1B).

When MCF-7 cells were co-exposed to ZEA and AFB1, AFB1 at 100 nM significantly shifted the dose response curve downwards (Figure 2A). Extra-sum-of-squares F test was performed to test if there is significant difference between the three dose response curves. Results showed that there were significant differences between the data sets ($F_{8, 70} = 99.57$, $p < 0.0001$). The best-fit values of the bottom, top, hill slope, half maximal effective concentration (EC₅₀) and r^2 values are shown in Supplementary Table 1. There were significant differences between all best fit values except that of the hill slope. By inspecting the 95% CIs, the significances were given by the curve of ZEA + AFB1 100 nM as its 95% CIs did not overlap with those of ZEA and ZEA + AFB1 at 1 nM. Two-way ANOVA was further performed for selected doses of the mycotoxin mixtures to verify the results. There was a statistically significant interaction between the effects of ZEA and AFB1 on cell viability ($F_{4, 26} = 15.13$, $p < 0.0001$). With the addition of AFB1 100 nM, the cell viability in all groups of ZEA significantly decreased (Figure 2B).

BrdU incorporation was used for measuring the extent of DNA synthesis following exposure to the two mycotoxins. There was a significant interaction between ZEA and AFB1 on DNA synthesis ($F_{4, 27} = 3.791$, $p < 0.05$), and simple main effects analysis showed that DNA synthesis was significantly affected by both ZEA ($F_{2, 27} = 43.31$, $p < 0.0001$) and AFB1 ($F_{2, 27} = 81.39$, $p < 0.0001$). ZEA significantly increased the DNA synthesis at both 1 and 100 nM, except when AFB1 100 nM was added. AFB1 decreased the DNA synthesis in MCF-7 cells significantly only at 100 nM (Figure 3). The results from BrdU incorporation assay complement that of MTT assay.

To further corroborate these results, cell cycle analysis was performed in MCF-7 cells after 24-hour treatments of the two mycotoxins. No significant interactions were found for ZEA and AFB1 on percentage distribution of cells in G0 – G1 phases ($F_{4, 27} = 0.1131$, $p = 0.98$), S

phase ($F_{4, 27} = 0.3209$, $p = 0.86$) and G2 – M phases ($F_{4, 27} = 0.1873$, $p = 0.94$). Percentage of cells in G0 – G1 phases was significantly decreased by about 30% and that in S phase was doubled by ZEA but no effect was observed for AFB1 (Figures 4A and 4B). Significant difference was also detected for ZEA on percentage of cells in G2 – M phases ($F_{2, 27} = 7.977$, $p = 0.0019$), but individual differences were not detected which might be due to the large variations within groups. On the other hand, two-way ANOVA results indicated that AFB1 caused a slight decline in percentage of cells in G0 – G1 phases ($F_{2, 27} = 3.541$, $p = 0.043$). However, individual differences among the treatment groups were not detected by Tukey's post hoc test. AFB1 also had no effect on percentage of cells in S phase ($F_{2, 27} = 2.948$, $p = 0.070$) and G2 – M phases ($F_{2, 27} = 0.7629$, $p = 0.48$).

3.2 Differential Expression Profiles of Breast Carcinogenesis Genes.

Expression profiles of 84 genes frequently involved in breast carcinogenesis after different treatments were examined by a commercially available qPCR array. The genes included in the qPCR array were categorized into 11 functional groups (cell cycle, apoptosis, DNA damage, signal transduction and the others) based on their known functions (Supplementary Table 2). From the analysis of qPCR array, it was found that expression of 32 genes were increased or decreased by more than 2-fold in the groups of ZEA 100 nM and ZEA 100 nM + AFB1 1 nM (Table 1). Expression of 36 genes were affected by the treatment ZEA 100 nM + AFB1 100 nM (data not shown). Only expression of 5 genes were affected by AFB1 1 nM and AFB1 100 nM, respectively. Excluding the genes with relatively low expression in all of the groups (C_T values from 30 to more than 35), a total of 36 genes were affected by different treatments for more than 2-fold, and are summarized below in Table 1 with their known functions and responsiveness to E_2 based on available literature. Figure 5A shows a Venn diagram analysis of the genes 24 hours post ZEA and/or AFB1 exposure and illustrates the common and unique gene expression changes among the treatment groups. ZEA affected the expression of a wide variety of genes related to breast carcinogenesis, and the effects were similar to that of E_2 in breast, uterus or ovary. The modulation of gene expression was similar with the addition of AFB1. AFB1 alone seemed to only affect the expression of cyclindependent kinase inhibitor 2A (CDKN2A), GLI family zinc finger 1 (GLI1) and epidermal growth factor (EGF). Cluster analysis also revealed that the expression profiles of the two mixtures (ZEA 100 nM + AFB1 1 nM and ZEA 100 nM + 100 nM) were the closest to the expression profile of ZEA 100 nM than to those of AFB1 (Figure 5B). Exposure to AFB1 alone affected gene expression to a much less extent, as the expression profiles of the two concentrations of AFB1 (1 nM and 100 nM) were similar to that of the untreated control. This might indicate that ZEA's carcinogenic potential was superior to AFB1, though the reasons for this are not clear.

To verify the similarity of expression profiles of different treatments, non-parametric Spearman correlation analyses were performed (Supplementary Figure 1). Sequential Bonferroni correction for the critical p value was implemented due to comparison of multiple tests (total 10 tests). After correction, strong positive associations were found between the pairwise groups of ZEA 100 nM and ZEA 100 nM + AFB1 1 nM ($r_s = 0.84$, $p < 0.0001$, Supplementary Figure 1C); ZEA 100 nM and ZEA 100 nM + AFB1 100 nM ($r_s = 0.81$, $p < 0.0001$, Supplementary Figure 1D); and ZEA 100 nM + AFB1 1 nM and ZEA 100

nM + AFB1 100 nM ($r_s = 0.79$, $p < 0.0001$, Supplementary Figure 1J), suggesting the expression profiles between these three treatments were similar. Significant correlation was also found between the AFB1 1 nM and AFB1 100 nM ($r_s = 0.34$, $r_s^2 = 0.12$, $p < 0.001$, Supplementary Figure 1E). However, the low values of r_s^2 implied that the association between these groups was weak.

To confirm array data, qPCR was performed for 5 selected genes with different functions (Figure 6). No interactions were found between effects of ZEA and AFB1 on expression of these genes. All genes were up-regulated ($p < 0.0001$) when cells were exposed to ZEA 100 nM, while AFB1 only had effect on expression of MKI67 ($F_{2, 18} = 3.909$, $p < 0.040$, Figure 6A). E_2 was included in the validation and the magnitudes of effects by ZEA were comparable to that of E_2 . The results of qPCR agreed with that of the qPCR array.

Combined Effects of ZEA and AFB1 on Cell Proliferation Through ER α , GPER and MAPK pathways.—The mRNA transcript levels of ER α and GPER were first determined after exposure to ZEA and AFB1 for 24 hours. No significant interactions were found between effects of ZEA and AFB1. But both transcript levels of ER α (Figure 7A) and GPER (Figure 7B) were significantly reduced by ZEA ($F_{1, 18} = 36.62$, $p < 0.0001$ for ER α ; and $F_{1, 18} = 170.4$, $p < 0.0001$ for GPER) and AFB1 ($F_{2, 18} = 8.565$, $p = 0.0024$ for ER α ; and $F_{2, 18} = 4.303$, $p < 0.030$ for GPER). The involvement of ER α and GPER in ZEA's activity was further confirmed by co-incubating MCF7 cells with inhibitors of ER α and GPER. As shown in Figure 8, only MPP completely abolished the proliferation of MCF-7 cells, suggesting that ER α was responsible for the proliferative effects by ZEA. Furthermore, the magnitude of proliferation by ZEA was reduced when G-15 and JNK inhibitor II were added (from 3-fold without inhibitor to 1.6 and 1.7 fold respectively), implying that GPER and JNK might be partially involved in the proliferative stimulation by ZEA.

When MCF-7 cells were co-incubated with ZEA 100 nM + AFB1 100 nM without inhibitors, proliferative effects were not observed. Surprisingly, when inhibitors of MAPKs (PD98059, SB203580 and JNK inhibitor II) were added, the proliferative effects by the mixture ZEA 100 nM + AFB1 100 nM were comparable to that of ZEA 100 nM alone. The results suggested the direct involvement of MAPKs for the cytotoxic effects caused by AFB1. Besides, significances between the control and AFB1 100 nM groups disappeared when inhibitors were added. It might be the results of low absorbance of both control and AFB1 100 nM groups that made comparisons impossible.

4. Discussion

This study is the first to report the effects of combination of ZEA and AFB1 on cell growth and cell cycle progression of human breast cancer cells. The concentrations of ZEA and AFB1 used in this study (1 nM to 100 nM) are highly relevant to real-life exposure and people might be adversely affected by ZEA or AFB1 in normal daily life. Our data showed that ZEA promotes cell growth and cell cycle progression at nano-molar range, which was in agreement with previous studies (Abassi et al., 2016; Dees et al., 1997; Khosrokhavar et al., 2009; Minervini et al., 2005; Parandin et al., 2015; Yu et al., 2005). Exposure to ZEA at

nano-molar range significantly increased DNA synthesis, percentage of cells entering proliferative phases (S phase and G2 – M phases) and eventually cell proliferation. On the other hand, AFB1 alone caused a cytotoxic effect as determined by MTT assay and significantly reduced DNA synthesis at 100 nM. AFB1 100 nM also diminished the positive effects by ZEA on cell growth, DNA synthesis, and cell growth. However, AFB1 had minimal effects on cell cycle progression. The decrease in cell viability and BrdU incorporation might be due to the cytotoxic effects of AFB1 such as increased reactive oxidative stress, DNA damage, inhibition of DNA and protein synthesis, and programmed cell death at micro-molar range (IARC, 2002). The lack of significant effects on cell cycle progression might be because of the fact that cells in the untreated group were also in the quiescence stage so that no differences could be detected.

Moreover, our study gives an insight into how ZEA and AFB1 could modulate the growth of breast cancer cells by alternating expression of genes related to breast carcinogenesis. The qPCR array showed that the expression profiles of breast cancer genes were entirely different between cells exposed to ZEA and AFB1. ZEA affected expression of a much larger number of breast cancer genes compared to AFB1, and the effects of ZEA were similar to that of E₂, which were consistent with a previous study by Parveen et al (2009). This might indicate that ZEA's carcinogenic potential was superior to AFB1, though the reasons for this are not clear. Furthermore, when cells were co-exposed to ZEA and AFB1, the expression of genes were more similar to that of ZEA than AFB1, implying that when breast cancer cells were co-exposed to the two mycotoxins, the cytotoxic effects of AFB1 could not completely abolish the growth promoting effects of ZEA.

ZEA has long been recognized as an estrogenic compound. It is widely accepted that it exerts its effects by binding to nuclear ERs and subsequently activates transcription of estrogenic genes. Down-regulation of hormone receptor by an agonist is considered to be an additional hallmark of receptor activation in cancer cells (Albanito et al., 2007; Santagati et al., 1997). For example, protein levels of ER α were diminished by incubating estrogen-responsive cells with E₂ (Albanito et al., 2007; Santagati et al., 1997), and the induction of ER α is associated with a decrease in mRNA levels of ER α (Saceda et al., 1989). Consequently, the effects of ZEA and AFB1 on mRNA levels of ER α and GPER were studied. Consistent with the current knowledge of ZEA, our data has shown that ER α was responsible for most of the activities by ZEA. ZEA significantly reduced the transcript levels of ER α and GPER, suggesting ZEA could exert effects on MCF-7 cells through activating membrane-bound GPER in addition to classic ER α , as demonstrated by other studies (Jefferson et al., 2002; Kuiper et al., 1998; Le Guevel and Pakdel, 2001; Li et al., 2012; Miksicek, 1994). AFB1 also seems to reduce the transcript levels of ER α and GPER as analyzed by two-way ANOVA, although significant differences were not detected by post-hoc Tukey's test. The reduction might be the result of cytotoxicity of AFB1, as AFB1 could inhibit DNA and protein synthesis at relatively high doses (IARC, 2002). However, the reduction was not comparable to that of ZEA. Other than being a hallmark of receptor activation, down-regulation of ER α and GPER could also disturb the normal hormone function. The involvement of ER α and GPER in ZEA's activity was further confirmed by co-incubating MCF7 cells with inhibitors of ER α and GPER. Inhibition of ER α

significantly eliminated the ZEA-induced proliferation of MCF-7 cells, whereas inhibition of GPER partially suppressed the growth promoting effects of ZEA.

Together with a few previous studies carried out by other scientists (Ahamed et al., 2001; Li et al., 2012; Parveen et al., 2009), it was suggested that ZEA might also provoke breast cancer cell growth through rapid cellular signaling in addition to the classical nuclear ER pathway. MAPK signaling pathways (ERK, p38 and JNK pathways) are important for transducing extracellular stimuli to elicit cellular biological responses, and are critical in regulating complex cellular events such as proliferation, apoptosis, development, differentiation and transformation (Zhang and Liu, 2002). To examine the possible regulation of cytosolic MAPK signaling pathways by ZEA and AFB1, cells were co-treated with inhibitors of MAPKs in addition to ZEA and AFB1. The results have shown that ZEA-induced proliferation was partially reduced by inhibiting JNK. Although it is believed that JNK pathways respond to stress stimuli, Pedram et al. (1998) showed that JNK could be cross-activated by ERK, and JNK was the final mediator of ERK for vascular endothelial cell growth factor-induced endothelial cell proliferation. As a result, it is also possible that JNK was partially involved in the proliferative effects by ZEA.

Moreover, it was observed that with the addition of inhibitors of MAPKs, the negative effects on viability by AFB1 were counteracted. Other than regulating cell proliferation, MAPKs especially JNK and p38 play a major role in apoptosis and cell survival (Zhang and Liu, 2002). A closely related aflatoxin AFG1 (AFG1) was recently found to activate ERK, JNK and p38 in human adenocarcinomic alveolar basal epithelial cells. The authors also concluded that AFG1 trigger apoptosis by mediating the JNK and p38 signaling (Shen et al., 2014). Since AFB1 and AFG1 are similar in structure, AFB1 might also exert its partial cytotoxic effect through activation of these MAPK pathways.

5. Conclusion

In light of the results obtained, our data suggested the potential interactive effects of ZEA and AFB1 on breast cancer cell proliferation and cell cycle progression. A summary of the toxic mechanisms of ZEA on breast cancer cell growth with antagonistic effects posted by AFB1 was depicted in Figure 9. Our results confirmed the growth promoting properties of ZEA and the mechanistic actions of ZEA were similar to that of endogenous E₂, whereas those of AFB1 were mainly due to its cytotoxic effects. This study suggested that endocrine-disrupting mycotoxins that co-occur in human food can interact and influence the proliferation of tumor cells in humans. Future work on the interactive effects of these endocrine-disrupting mycotoxins or other xenoestrogens in normal human breast tissue cells and in breast tumors is warranted, which will contribute to improved risk assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ZEA and AFB1 produced significant interactive effects on cell growth and cell cycle.
- ZEA promoted growth, DNA synthesis and cell cycle progression.
- AFB1 was cytotoxic and counteracted the effects of ZEA.
- ER α , GPER and MAPK were found to be responsible for ZEA's effects on cell growth. While MAPK pathways might be involved in cytotoxic effects by AFB1.

Figure 1.

Effect of exposure to (A) ZEA and (B) AFB1 alone on cell viability in MCF-7 cells. MCF-7 cells were exposed to ZEA (10^{-12} to 10^{-5} M) and AFB1 (10^{-12} to 10^{-6} M) individually for 5 days. E₂ at 1 nM was included as positive control. Cell viability after treatments was measured by MTT assay and data are shown as mean $\pm$ 95% CI of 4 independent experiments and each experiment with 6 replicates. One-way ANOVA was performed for the comparison of effects by different concentrations of individual mycotoxins and untreated control. **** indicates $p < 0.0001$ compared to the control, as determined by Tukey's post hoc test and two sample t-test.

Figure 2.

Combined effects of ZEA and AFB1 on MCF-7 cell viability. Cells were exposed to ZEA (10^{-12} to 10^{-6} M) alone or together with AFB1 10^{-9} M (1 nM) or 10^{-7} M (100 nM) for 5 days, and viability was measured by MTT assay. (A) Dose response curve of ZEA + AFB1 100 nM shifted downwards from the curves of ZEA alone and ZEA + AFB1 1 nM. (B) Combined effects on cell viability by selected doses of ZEA and AFB1 are shown. The data represent mean $\pm$ 95% CI of 4 independent experiments and each experiment with 6 replicates. Two-way ANOVA was performed and treatment groups with different case letters indicates that they are significantly different, $p < 0.05$, as determined by Tukey's post hoc test.

Figure 3.

Effects of ZEA and AFB1 on DNA synthesis. MCF-7 cells were exposed to ZEA and AFB1 individually and in combination for 5 days, and the extent of DNA synthesis was measured by BrdU incorporation assay. E₂ at 1 nM was included as positive control. The results here represent mean $\pm$ 95% CI of 4 independent experiments and each experiment with triplicates. Twoway ANOVA was performed and treatment groups with different case letters indicates that they are significantly different, $p < 0.05$, as determined by Tukey's post hoc test. * indicates $p < 0.05$ compared with the untreated control, analyzed by two sample t-test.

Figure 4.

Cell cycle analysis was carried out after MCF-7 cells were exposed to mycotoxins for 24 hours. Cells were collected and stained with PI after fixation, and the nucleic acid content was measured by flow cytometer. E₂ at 1 nM was included as positive control. Percentage distribution of cells in (A) G0 – G1 phases, (B) S phase and (C) G2 – M phases are shown as mean ± 95% CI of 4 independent experiments and each experiment with duplicates. Two-way ANOVA was performed and treatment groups with different case letters indicates that they are significantly different, $p < 0.05$, as determined by Tukey's post hoc test. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with the untreated control, analyzed by two sample t-test. G0, resting phase; G1, growth 1 phase; G2, growth 2 phase; M phase, mitosis; S phase, synthesis phase.

A

B

Figure 5.

(A) Venn diagram analysis and (B) Functional cluster analysis. Significant differential gene expression (≥ 2 folds) between mycotoxins treated cells and untreated cells were identified by the RT² Profiler PCR Array Data Analysis. Final filtered gene lists generated were input into R software v.3.3.2 (<https://www.r-project.org/>) (R Core Team, 2016) in combination with RStudio v.1.0.136 (RStudio, Inc., Boston, MA, USA) to create Venn diagrams. Different mycotoxin treatments and untreated control were clustered based on the expression profiles of 84 genes. Expression were normalized with 3 internal control genes β -actin (ACTB), β -2microglobulin (B2M) and large ribosomal protein P0 (RPLP0). Red colour represents high expression of the genes while green colour represents minimal gene expression. Breast cancer genes were categorized into 11 functional groups according to the qPCR array manufacturer's information. A list of the genes analyzed in the array with their name and functions is given in Supplementary Table 2. Treatments: 0, untreated control; A9, AFB1 1 nM; A7, AFB1 100 nM; Z7, ZEA 100 nM; Z7A9, ZEA 100 nM + AFB1 1 nM; Z7A7, ZEA 100 nM + AFB1 100 nM. Genes that have more than 1 function are only shown once in the first group they appear. E to M transition, Epithelial to mesenchymal transition.

Figure 6.

Validation of qPCR array by qPCR. (A) antigen identified by monoclonal antibody Ki67 (MKI67); (B) c-Myc (MYC); (C) breast cancer 1, early onset (BRCA1); (D) baculoviral IAP repeat containing 5 (BIRC5); and (E) progesterone receptor (PGR) were found to be modulated by more than 2 folds in the qPCR array, and were chosen for validation by qPCR. Expression levels of the genes were normalized validation by qPCR. Expression levels of the genes were normalized with internal reference control RPLP0, and were reported as relative expression levels here. E₂ at 1 nM was included as positive control. The results represent

mean $\pm$ 95% CI of 4 independent experiments and each experiment with triplicates. Two-way ANOVA was performed and treatment groups with different case letters indicates that they are significantly different, $p < 0.05$, as determined by Tukey's post hoc test. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with the untreated control, analyzed by two sample t-test.

Figure 7.

Effects of ZEA and AFB1 on mRNA levels of (A) ERα and (B) GPER. The mRNA levels were normalized with internal reference control RPLP0, and were reported as relative expression levels here. E₂ at 1 nM was included as positive control. The results here represent mean ± 95% CI of 4 independent experiments and each experiment with triplicates. Two-way ANOVA was performed and treatment groups with different case letters indicates that they are significantly different, $p < 0.05$, as determined by Tukey's post hoc test. ** indicates $p < 0.01$ compared with the untreated control, analyzed by two sample t-test.

Figure 8.

Viability of MCF-7 cells exposed to ZEA and AFB1 for 5 days with or without cotreatment with MPP, G-15, PD 98059, SB 203580 and JNK inhibitor II (inhibitors of ER α , GPER, MEK, p38 and JNK respectively). Viability was calculated as the optical density of the sample minus the background compared to that of the control in each inhibitor group. E₂ at 1 nM was included as positive control. The results here represent mean $\pm$ 95% CI of 4 independent experiments and each experiment with 6 replicates. One-way ANOVA was performed for each inhibitor group and * represents significant difference with the control in each group ($p < 0.05$), as determined by Tukey's post hoc test.

Figure 9.

Summary of the toxic mechanisms of ZEA on breast cancer cell growth with antagonistic effects posted by AFB1. 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; AFB1, aflatoxin B1; *c-myc*, protein coded by myelocytomatosis oncogene; CYP, cytochrome P450; DNA, deoxyribonucleic acid; E₂, 17 β estradiol; EREs, estrogen response elements; ERs, estrogen receptors; G2/M phase, premitotic/mitotic phase; JNK pathway, c-Jun N-terminal kinase pathway; MAPK1/3 pathway, mitogen-activated protein kinase1/3 pathway; mER α , membrane-bound estrogen receptor α ; mGPER, membrane-bound G protein-coupled estrogen receptor 1; MYC,

myelocytomatosis oncogene; nER α , nuclear estrogen receptor α ; p38 pathway, p38 mitogen-activated protein kinase pathway; PGR, progesterone receptor; S phase, synthesis phase; *Src*, Proto-oncogene tyrosine-protein kinase *Src*.

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Table 1
List of up-regulated and down-regulated genes (more than 2-fold) with their annotated functions and relationship with E₂.

Symbol ^a	Fold regulation compared to untreated control					Cell cycle	Apoptosis	DNA damage	Signal transduction	Transcription factors	Xenobiotic transport	E to M transition	Angiogenesis	Adhesion	Proteolysis	Tumor classification	E ₂ responsiveness ^b	
	ZEA 100 nM	AFB1 1 nM	AFB1 100 nM	ZEA 100 nM + AFB1 1 nM	ZEA 100 nM													
BCL2	2.42	1.16	1.20	2.56	1.96	0	0		0					0			+	+
CCND2	-57.95	1.24	1.51	-7.03	-7.68	0											+	+
CCNE1	1.42	1.17	1.01	1.41	2.02	0			0								+	+
CDKN1C	-2.92	1.07	1.11	-3.58	-2.47	0											-	-
CDKN2A	-6.63	2.40	2.98	-1.38	-1.32	0	0							0			-	-
MDM7	2.59	-1.03	-1.15	2.46	2.26	0											+	+
MYC	5.29	-1.05	1.01	4.68	4.36	0				0							+	+
RASSF1	2.79	1.31	1.36	2.85	2.91	0											+	+
IGF1	-33.71	-1.15	-1.30	-4.02	-8.33		0		0								+	+
IL6	-1.72	1.35	2.07	-1.71	-5.90		0						0				-	-
NME1	3.43	-1.19	-1.06	4.09	3.28		0		0								+	+
RARB	-2.09	-1.02	-1.03	-2.08	-2.01		0			0							-	-
TP73	1.40	1.35	1.24	2.27	2.64		0	0	0	0							+	+
TWIST1	-6.06	-2.02	1.34	1.17	-3.86		0					0					-	-
BRCA1	2.19	1.01	-1.24	2.35	2.80			0	0							0	+	+
BIRC5	3.43	-1.03	-1.09	3.19	2.96				0							0	+	+
GLI1	1.37	2.47	2.56	3.60	2.48				0								+	+
IGFBP3	-6.95	-1.14	1.12	-5.54	-5.73				0								-	-
NOTCH1	1.27	1.79	1.44	1.64	2.24				0	0		0				0	+	+
NR3C1	-2.54	1.11	-1.07	-2.82	-2.18				0	0							-	-
PGR	9.74	1.04	1.10	11.38	10.47				0	0							+	+
SNAIL2	-2.83	1.39	1.16	-2.69	-2.48												-	-
XBP1	3.86	1.08	1.15	4.87	4.13								0			0	+	+
EGF	1.38	2.16	2.33	3.93	2.71									0			+	+
SERPINE1	1.43	1.08	1.49	2.20	3.53									0			+	+
THBS1	-2.67	-1.05	1.11	-1.69	-2.06									0			-	-
SLIT2	-4.35	1.06	1.63	-3.60	-3.95									0			-	-
CST6	-3.32	1.61	1.27	1.04	-1.05										0		-	-
CTSD	2.80	1.03	-1.01	2.93	2.62									0			+	+
MMP2	2.13	1.10	-1.34	1.26	-1.29										0	0		
GRB7	-2.51	1.14	1.04	-2.17	-2.29											0	+	+

Symbol ^a	Fold regulation compared to untreated control					Cell cycle	Apoptosis	DNA damage	Signal transduction	Transcription factors	Xenobiotic transport	E to M transition	Angiogenesis	Adhesion	Proteolysis	Tumor classification	E2 responsiveness ^b	
	ZEA 100 nM	AFB1 1 nM	AFB1 100 nM	ZEA 100 nM + AFB1 1 nM	ZEA 100 nM + AFB1 100 nM													
KRT5	3.61	1.21	-1.11	5.69	2.97											0		
SLC39A6	2.42	-1.05	-1.03	2.01	2.14											0		
TFF3	3.37	-1.01	1.15	3.73	3.45											0		+

^aFor the names of the genes please refer to Supplementary Table 2.

^bResponsiveness to E2 is based on available literature search in human breast, uterus and ovary. + suggests the genes are up-regulated by E2; – suggests the genes are down-regulated by E2; there is no conclusive information available for the responsiveness of the gene to E2 when no + or — is indicated. E to M transition, epithelial to mesenchymal transition.