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Comparative acute and combinative toxicity of aflatoxin B_1 and fumonisin B_1 in animals and human cells

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Abstract

Aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) are important food-borne mycotoxins. The co-contamination of food stuffs with these two mycotoxins is well known and has been possibly implicated in the development of human hepatocellular carcinoma in high risk regions around the world. In this study the acute and combinative toxicity of AFB_1 and FB_1 were tested in F-344 rats, mosquitofish (*Gambusia affinis*), immortalized human hepatoma cells (HepG2) and human bronchial epithelial cells (BEAS-2B). Preliminary experiments were conducted in order to assess the acute toxicity and obtain LD_{50}, LC_{50} and IC_{50} values for individual toxins in each model, respectively. This was followed by testing combinations of AFB_1 and FB_1 to obtain LD_{50}, LC_{50} and IC_{50} values for the combination in each model. All models demonstrated a significant dose response in relation to toxin treatment. The potency of the mixture was gauged through the determination of the interaction index metric. Results of this study demonstrate that these two toxins interacted to produce alterations in the toxic responses with a strong additive interaction noted in the cases of F344 rats and mosquitofish. It can be gathered that this combination may pose a significant threat to public health and further research needs to be completed addressing alterations in metabolism and detoxification that may influence the toxic manifestations in combination. These results will provide foundational knowledge for future studies on long-term combinative toxic and health effects of these mycotoxins.

Keywords: Aflatoxin B_1; Fumonisin B_1; Rats; Mosquitofish; HepG2; BEAS-2B; Acute; Combinative; Toxicity

1. Introduction

Toxic fungal metabolites, mycotoxins, are structurally diverse compounds, which represent the most important category of biologically produced natural toxins relative to human health and economic impact worldwide (Cole and Cox, 1981; Ciegler et al., 1981). Spurred by the discovery of aflatoxin in the 1960s the first cases of mycotoxicoses were noted leading to the identification of more than 100 toxigenic fungi and in excess of 300 mycotoxins worldwide (Sharma and Salunkhe, 1991; Miller and Trenholm, 1994). These mycotoxins display diverse chemical structures accounting for their differing biological properties and effects. Depending upon the toxins precise biochemical nature, it may have any of a number of toxic properties including being carcinogenic, tetratogenic, mutagenic, oestrogenic, neurotoxic, or immunotoxic.

Aflatoxins (AFs) represent a group of closely related difuranocoumarin compounds produced as secondary

Abbreviations: AFs, aflatoxins; AFB_1, aflatoxin B_1; BEAS-2B, immortalized human bronchial epithelial cells; FNs, fumonisins; FB_1, fumonisin B_1; HepG2, immortalized human hepatoma cells; IC_{50}, concentration to cause 50% growth inhibition; LC_{50}, lethal concentration to cause 50% mortality of test animals; LD_{50}, lethal dose to cause 50% mortality of test animals.

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fungal metabolites of the common molds *Aspergillus flavus*, *Aspergillus parasiticus* and to a lesser extent *Aspergillus nominus*. Three strains of *Aspergillus* have been found from which four major AFs (AFB_1, AFB_2, AFG_1, and AFG_2) are produced. AFB_1 is the most prevalent and toxic of the AFs, with acute toxicity demonstrated in all species of animals, birds, and fish tested resulting in LD_{50} values ranging from 0.3 to 9.0 mg/kg body weight. AFB_1 is also known as being one of the most potent genotoxic agents and hepatocarcinogens identified (Busby and Wogan, 1984; Sharma and Salunkhe, 1991; Miller and Trenholm, 1994; Wang et al., 1998).

The toxicity and carcinogenicity of AFB_1 is thought to be directly linked to its bioactivation, resulting in a highly reactive AFB_1 8,9-epoxide (AFBO). This bioactivation of AFB_1 occurs primarily by a microsomal cytochrome P450 (CYP450) dependent epoxidation of the terminal furan ring of AFB_1 and is responsible for binding to cellular macromolecules such as DNA, RNA and other protein constituents (Massey et al., 1995; Wang and Groopman, 1999). Damage to and necrosis of hepatocytes as well as other metabolically active cells is believed to be the result of this process (Eaton and Groopman, 1994).

The fumonisins (FNs) are a group of structurally related polar metabolites produced by *Fusarium verticillioides*, *Fusarium moniliforme*, *Fusarium proliferatum* and other species to a lesser extent. Their structures are based on a hydroxylated hydrocarbon chain which contains methyl and either amino (B_1 and B_2) or acetyl groups (A_1 and A_2). Fumonisin B_1 (FB_1) was first isolated in 1988 (Gelderblom et al., 1988; Bezuidenhout et al., 1988). It is the most abundantly produced of the FNs in contaminated foods and feeds, found primarily as a contaminant of the corn constituent, and is the most toxicologically significant (Riley et al., 1993).

FB_1 has been linked to several human and animal disease outbreaks worldwide displaying the unique biological effects of this chemical. These include species, sex, and tissue specificity resulting in neurotoxicity, hepatotoxicity, nephrotoxicity, immune suppression, immune stimulation, developmental abnormalities, liver tumors, kidney tumors, and other abnormalities (Marasas et al., 1988; Harrison et al., 1990; Voss et al., 2001; Howard et al., 2001; for a review see Marasas, 1997; Riley, 1996).

Recently, there have been numerous in vivo and in vitro studies which provide support to the hypothesis that the biochemical mechanism of action involves altered sphingolipid metabolism. FB_1, which is structurally similar to sphinganine (Sa), inhibits the enzyme sphingosine [sphinganine] *N*-acyltransferase (ceramide synthase) (Wang et al., 1991). Ceramide synthase is a key enzyme responsible for the acylation of Sa in the de novo synthesis of sphingolipids and the reacylation of sphingosine (So) (Merrill et al., 1993). The inhibition of ceramide synthase by FB_1 results in changes in the major pools of sphingolipids, including increases in free sphingoid base metabolites, decreased biosynthesis of ceramide and/or other lipids containing ceramide, and disruption of fatty acid and glycerophospholipid metabolism (Riley et al., 1993; Gelderblom et al., 1992; for a review see Creppy, 2002). The effects of FB_1 consequent to the above actions are complex and may differ between tissue, species, and sex (Turner et al., 1999).

Co-exposure to multiple mycotoxins is a cause of concern because so many have been shown to be potent toxic agents with diverse effects and a synergetic nature. It is logical to raise this issue because any single compound may effect dissimilar reactions within a biological system, while displaying antagonistic, additive, or synergistic interactions with other compounds (Carpenter et al., 1998). However, little attention has been paid in study of combinative toxic effects of exposure to multiple mycotoxins, which may be more potent and cause more damage to human health. The nature of co-existence of many types of mycotoxins in complex environmental samples, such as food and water, has been reported worldwide. How these mycotoxins affect human health in combination is largely unknown. In this study, we extend our research efforts to test combinative toxicity of the AFB_1 and FB_1 combination in animals (F344 rats and mosquitofish) and human cells (BEAS-2B and HepG2).

2. Materials and methods

2.1. Materials

Mycotoxins selected for this study AFB_1 and FB_1 were either purchased from Sigma Chemical Co. (St. Louis, MO) or were kindly provided by research units of the US Food and Drug Administration. Purity of each toxin (>98%) was tested with the appropriate analytical tool: AFB_1 with high-performance liquid chromatograph (HPLC)-UV detection and FB_1 with HPLC-fluorescence detection after derivatization with *o*-phthaldialdehyde (OPA, Sigma). Stock solutions (25 mg/ml) were made with dimethylsulfoxide (DMSO) and kept under argon. Human hepatoma cell line ($HepG_2$) and human bronchial epithelial cell line (BEAS-2B) were purchased from ATCC (Manassas, VA). All other chemicals and reagents were purchased commercially at the highest degree of purity available.

2.2. Animals and treatment

2.2.1. Fischer 344

Young male Fischer 344 rats (90–110 g) were obtained from Harlan Lab Animals Inc. (Indianapolis, IN) a week before experiments were initiated and housed individually in stainless steel cages under controlled temperature (22 °C ± 1 °C), light (12 h light-dark cycle), and humidity (50% ± 10%). NIH open formula diet (NIH-07 Rat and Mouse Feed; Zeigler Bros., Inc.; Gamers, PA) and distilled water were supplied ad libitum. The acute toxicity study for individual mycotoxins was performed using the method described by Horn (1956). Briefly, F344 rats were randomly divided into 5–7 groups of five animals. One group was only given solvent vehicle (DMSO) and used as the control. The other groups were orally administered mycotoxin at 1.0, 2.15, 4.64, 10.0, or 46.4 mg/kg body weights, respectively. The study was performed over 7 days. Animals were carefully observed after treatment and symptoms of toxicity were recorded. Animals that died during the experiment or were euthanized by halothane (2-brome-2-chloro-1,1,1,-trifluoroethane) inhalation after the experiment were necropsied. The major organs were excised and fixed in 10% buffered formalin for histopathological evaluations.

The combinative toxicity study for mycotoxin mixtures was performed using the method described by Cornfield (1964). In this study, F344 rats were randomly divided into seven groups. Each group included 8 animals. One group was only given solvent vehicle (DMSO) and used as the control. The other groups were administered by the same gavage with various fractions of the derived LD_{50} for each mycotoxin, respectively. The study was done over 14 days. Animals were carefully observed after treatment and symptoms of toxicity were recorded. Animals that died during the 14-day experiment or were euthanized by halothane (2-brome-2-chloro-1,1,1,-trifluoroethane) inhalation after the experiment were necropsied. The major organs were excised and fixed in 10% buffered formalin for histopathological evaluations.

2.2.2. Mosquitofish

Mosquitofish (*Gambusia affinis*) (6 months old) were purchased from Ken's Hatchery and Fish Farm, Inc. (Alapaha, GA) or Carolina Biological Supply Co. (Burlington, NC) three weeks before experiments were performed. After arrival, fish were maintained in a 350–750 l aquaria (to maintain a minimal fish density), filled with sea salt-buffered distilled water (60 mg/l), equipped with seasoned biological filters, and underwent quarantine procedures (treated once with trisulfa or every other day for six days with supersulfa). Goldfish flake fish food (Aquaria Pharmaceuticals, Inc., Chalfont, PA) was daily supplied ad libitum. After a week adaptation, fish were further separated according to their gender into 40-l aquaria under similar condition. Healthy fish (half male and half female) were randomly assigned into 3-l glass aquaria at the third week and were treated with various concentrations of individual toxins (0.1–10.0 mg/l) or combinations (1/8–1.0 LC_{50}) dissolved in water after one-day adaptation. The treated and control fish were observed for 5-days with morbidity and mortality recorded. The results were analyzed and LC_{50} determinations made by probit analysis.

2.3. Human cell lines

The $HepG_2$ and BEAS-2B cells were purchased from ATCC (Manassas, VA). $HepG_2$ cells were grown in RPMI-1640 media (ATCC, Manassas, VA), supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), at 37 °C in humidified 5% CO_2. BEAS-2B cells were maintained in LHC-9 media consisting of recombinant epidermal growth factor (0.5 ng/ml), hydrocortisone (0.5 μg/ml), insulin (0.005 mg/ml), bovine pituitary extract (0.035 mg/ml), ethanolamine (500 nM), phosphoethanolamine (500 nM), transferrine (0.01 mg/ml), 3,3′,5-triiodothyronine (6.5 ng/ml), epinephrine (500 ng/ml), retinoic acid (0.1 ng/ml) and trace elements. The bottom of the flask was covered with an appropriate quantity of coating medium derived from LHC basal medium (1 l) and 100 ml of 0.1% bovine serum albumin (Sigma, St. Louis, MO), 10 mg of human fibronectin (Sigma, St. Louis, MO), 30 mg of vitrogen 100 (Sigma, St. Louis, MO) to which the cell monolayer could adhere. Cytotoxicity of mycotoxins and their mixtures on $HepG_2$ and BEAS-2B cells was determined by PreMix WST-1 cell proliferation assay system (Takara Bio Inc., Shiga, Japan). This method assesses the ability to convert tetrazolium salts to formazan dye by the succinate-tetrazolium reductase, which exists in mitochondrial respiratory chain and is active only in viable cells. Freshly collected $HepG_2$ and BEAS-2B were seeded at 10^4 cells per well in octuplicate in 96-well tissue culture plates (Falcon, Franklin Lakes, NJ) and allowed to attach for 24-h to obtain a monolayer culture. Culture media were replenished with RPMI-1640 for $HepG_2$ and LHC-9 for BEAS-2B, supplemented with vehicle (0.01% DMSO) alone or various concentrations of an individual mycotoxin or mycotoxin mixture for 24-h. At the end of the designated reaction period, the culture medium was replaced with 200 μl of medium containing 10 μl of WST-1 solution and the plates were incubated for 4 h at 37 °C in humidified 5% CO_2. The absorbance was measured on an F-max microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 440 nm with background subtraction at 600 nm. Percent viability of the population of cells in each well was expressed as OD of treated cells/OD of control cells × 100%. All experiments were performed in triplicates and repeated at least twice.

2.4. Data analyses

Data for combinative acute toxicity testing in the F344 and mosquitofish models are expressed as the mortality rate. LD_{50} values and 95% confidence limit of individual toxins in the F344 model were obtained by Horn's method (Horn, 1956) of dosing in coordination with the moving-average interpolation method similar to that presented by Thompson (1947). Further individual and combinative LD_{50} and LC_{50} with 95% confidence limits were calculated according to the method of Cornfield (1964). Statistical analysis for data used software from SPSS 11.0 (SPSS, Inc., Chicago, IL). Data from combinative cytotoxicity studies in human cells were expressed as percent viability of the population of cells in each well, which was calculated from the formula: OD of treated cells/OD of control cells × 100%. The IC_{50} and 95% CI were also calculated using the probit analysis. One-way ANOVA and Dunnett's procedure were used to test significance of viability among controls and treated cells. Calculation of the interaction index (K) is described by Tallarida (2001) with slight modification. Briefly, in our model, (K) is determined by obtaining the Estimated [Theoretical] LD_{50}/Experimental [Measured] LD_{50}. If in the acute phase of the trials an individual mycotoxin is determined to be non-toxic through the dose range employed, the dose of that toxin is held constant over the regimen and is not included in the LD_{50} determination in that model (Tallarida, 2001).

3. Results

3.1. Acute toxicity of AFB_1 and FB_1 in F344 rats

Higher doses of AFB_1 (10.0 and 4.64 mg/kg bw) caused acute toxic symptoms immediately post-treatment. Mortality in treated animals occurred within 48 h post-treatment and within 72 h 100% mortality (5/5) was observed in animals treated with 10 mg/kg bw AFB_1. Mortality reached 100% in animals treated with 4.64 mg/kg bw AFB_1 at 96 h post-treatment. Twenty percent mortality was observed in animals treated with 2.15 mg/kg bw AFB_1 during the one-week study period. No mortality was observed in animals treated with the lowest dose (1.0 mg/kg bw) or in the vehicle control group. The LD_{50} was determined to be 2.71 mg/kg AFB_1 with the 95% confidence limit (CL) from 2.0 to 3.7 mg/kg bw.

In contrast to previous reports using intravenous or intraperitoneal routes (Voss et al., 2001; Wang et al., 1998), the acute oral toxicity of FB_1 in rats was quite low. No lethal effect was observed with doses of 1.0, 2.15, 4.64, 10.0, 21.5 and 46.4 mg/kg bw employed, although apparent toxic effects where noted (e.g., loss of feed consumption and lameness) in higher doses of FB_1 (21.5 and 46.4 mg/kg bw). We did not increase the dose of FB_1 for further experiments due to the high commercial cost of this mycotoxin.

3.2. Acute toxicity of AFB_1 and FB_1 in mosquitofish

The higher concentrations of AFB_1 (2150 and 1000 μg/l) caused acute toxic symptoms immediately post-treatment, such as reduction of activity and loss of righting reflex. Mortality appeared between 24 and 72 h after treatment with AFB_1. Within 72 h, 100% mortality (12/12) was observed in fish treated with 1000 and 2150 μg/l. Mortality

Table 1
Acute toxicity of AFB_1 in mosquitofish

Concentration (μg/l)	No. of fish	No. of deaths	Mortality (%)
Control	12	0	0
100	12	0	0
215	12	2	17
464	12	6	50
1000	12	12	100
2150	12	12	100

was also observed in the two lower concentrations of AFB_1-treated fish during 5-day study period (Table 1). The study yielded a LC_{50} value of 681 μg/l AFB_1 (Table 3).

The highest concentrations of FB_1 (10.0 and 4.64 mg/l) also caused acute toxic symptoms soon after treatment. Within 48 h mortality occurred in these groups with the concentration of 4.64 mg/l resulting in 50% mortality (6/12), and 10 mg/l only resulting in 67% mortality (8/12) during 5-day study period (Table 2). The 2.15 mg/l dose group recorded 17% mortality (2/12). The study yielded a LC_{50} value of 4.64 mg/l FB_1 (Table 3).

3.3. Cytotoxicity of AFB_1 and FB_1 in $HepG_2$ and BEAS-2B

The cytotoxic effects of AFB_1 at the doses of 0.01, 0.1, 1, 10, and 100 μM in the $HepG_2$ cells as measured by the tetrazolium dye-based WST-1 assay were assessed. At 24 h of exposure, 1 μM AFB_1 caused a marked decrease of the number of viable cells to 50% of the control level. The LC_{50} was estimated at 1.0 μM. Conversely, exposure of the BEAS-2B cells did not result in a dose-dependent viability response after treatment with the same dose range of AFB_1. Their viability was approximately 90% in all treated groups relative to controls.

The cytotoxic effects of FB_1 were assessed in $HepG_2$ cells at doses of 0.1, 1.0, 10.0, 100, and 500 μM. Overall the response to FB_1 in this cell line less than the expressed toxicity of AFB_1 examined. In fact, the lower concentrations of FB_1 (<10 μM) enhanced cellular proliferation, while only concentrations higher than 100 μM demonstrated any cytotoxic effect. The calculated IC_{50} for FB_1 to HepG2 cells was 399.2 μM with the 95% CL between 340.7 and 461.3 μM. The consequence of FB_1 in BEAS-2B cells was very similar and apparent from concentrations $\geqslant$100 μM. Based on these results, IC_{50} was calculated as 355.1 μM with the 95% CL from 255.85 μM to 526.13 μM (Table 4).

Table 2
Acute toxicity of FB_1 in mosquitofish

Concentration (μg/l)	No. of fish	No. of deaths	Mortality (%)
Control	12	0	0
464	12	0	0
1000	12	0	0
2150	12	2	17
4640	12	6	50
10,000	12	8	67

Table 3
Summary of LC_{50} determinations in mosquitofish by probit analysis

Mycotoxin	LC_{50} (μg/l)	95% CL (μg/l)
AFB_1	681	464–800
FB_1	4640	2150–10,000

Table 4
Summary of IC_{50} determinations in $HepG_2$ and BEAS-2B by probit analysis

Cell line	AFB_1 IC_{50} (μM)	AFB_1 95% CL (μM)	FB_1 IC_{50} (μM)	FB_1 95% CL (μM)
$HepG_2$	1.00	0.9–7.4	399.2	340.7–461.3
BEAS-2B	>100.0	ND	355.1	255.9–526.1

ND—not determined.

3.4. Combinative toxicity of binary mycotoxin mixture in F344 rats

Based on the determined LD_{50} values, experiments were carried out testing the acute combinative toxicity for these two toxins in F344 rats following the outlined procedures. On the basis of the LD_{50} for AFB_1, rats were treated orally with various doses of AFB_1 at 1.0, 3/4, 1/2, 3/8, 1/4, 1/8 LD_{50}, and 0 (control), respectively. Due to lower acute toxicity of FB_1 and lack of a LD_{50} value in F344 rats, the dose of 25 mg/kg bw was administered by gavage to all AFB_1-treated animals. Symptoms of acute toxic effects, such as depression and diarrhea, appeared within a few hours post-treatment in response to higher doses of the binary mixture. Mortality was recorded 72 h post-treatment while no effect was observed in the groups given the lowest doses of the combination. These results demonstrate that 25 mg/kg bw of FB_1 additively increased the mortality of rats caused by the acute toxicity of AFB_1 evidenced by the K value of 1.98 (Tables 5 and 9).

3.5. Combinative toxicity of binary mycotoxin mixture in mosquitofish

Experiments completed in the acute phase found that the mosquitofish were a sensitive fish model for our study following outlined procedures. Results of these

Table 5
Combinative toxicity of AFB_1 and FB_1 in F344 rats

Dose (LD_{50})	AFB_1 (mg/kg bw)	FB_1 (mg/kg bw)	No. of animals	No. of deaths	Mortality (%)
Control	0	0	8	0	0
1/8	0.34	25	8	0	0
1/4	0.68	25	8	0	0
3/8	1.02	25	8	2	25
1/2	1.36	25	8	2	25
3/4	2.03	25	8	8	100
1.0	2.71	25	8	8	100

Table 6
Combinative toxicity of AFB_1 and FB_1 toxin in mosquitofish

Concentration (LC_{50})	AFB_1 (μg/l)	FB_1 (μg/l)	No. of fish	No. of deaths	Mortality (%)
Control	0.0	0	12	0	0.0
1/8	85.1	580	12	3	25.0
1/4	170.3	1160	12	3	25.0
3/8	255.4	1740	12	9	75.0
1/2	340.5	2320	12	11	91.7
1.0	681.0	4640	12	12	100.0

experiments are shown in Table 6. The higher concentrations of these two toxins caused acute toxic symptoms, such as reduction of activity and loss of righting reflex. Mortality was initially observed in the lower concentration groups which subsided after two to three days and was superseded by elevated mortality rates in the higher concentration groups. As shown in Table 6, there was no dose-dependent mortality observed in the two lowest binary concentrations, however, an apparent dose response was found at higher concentrations. The estimated LC_{50} for the mixture of AFB_1 and FB_1 was 1341.7 μg/l with 95% CL of 968.7–1733.0 μg/l. The combinative toxicity index ($K = LC_{50}$ estimated/LC_{50} measured) was 1.98 (Table 9).

3.6. Combinative cytotoxicity of binary mycotoxin mixtures in human cells

Variable AFB_1 and FB_1 concentrations of 1.0, 3/4, 1/2, 3/8, 1/4, 1/8 IC_{50}, and 0 (control) were mixed and administered to HepG2 cells for 24 h following the procedures outlined in Section 2. After exposure the viability of the cells was measured and the results are presented in Table 7 and Fig. 1. Lower concentrations of combination of AFB_1 and FB_1 had no inhibitory effect on the cellular viability. In fact, these lower concentrations of combination increased the number of viable cells versus control values. Higher concentrations of these binary mycotoxins at 1.0, 3/4, and 1/2 IC_{50}, however, did show a greater reduction of the cellular viability of 32–69% of control values, respectively. The measured IC_{50} for this binary toxin mixture was 333.0 μM in HepG2 cells and the interaction index was

Table 7
Combinative toxicity of AFB_1 and FB_1 in $HepG_2$

Concentration (IC_{50})	AFB_1 (μM)	FB_1 (μM)	Binary mixture (μM)	Viability (% control ± SD, $n = 6$)
0	0	0	0	100 ± 7
0.13	0.13	49.9	50.0	112 ± 2
0.25	0.25	99.8	100.1	111 ± 12
0.38	0.38	149.7	150.1	113 ± 8
0.50	0.50	199.6	200.1	69 ± 11*
0.75	0.75	299.4	300.2	55 ± 10**
1.00	1.00	399.2	400.2	32 ± 8**

* $p < 0.05$ as compared with controls.
** $p < 0.01$ as compared with controls.

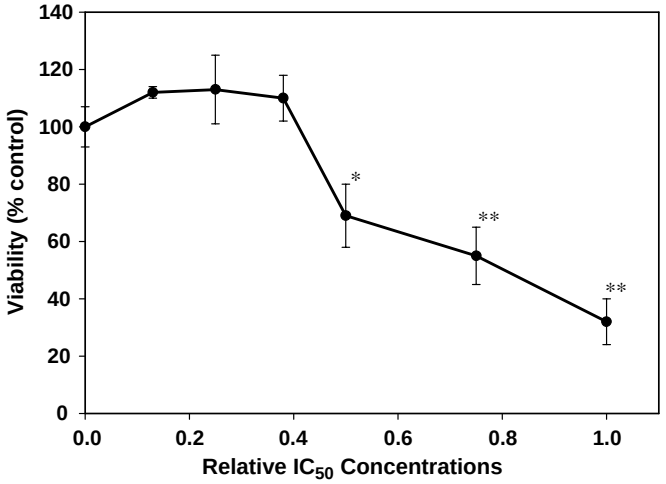

Fig. 1. Effect of AFB_1 and FB_1 on cellular viability in $HepG_2$ cell line. Values represent mean viability as % control ± SD ($n = 6$). The IC_{50} was determined by probit analysis to be 333.0 μM. *$p < 0.05$ as compared with controls; **$p < 0.01$ as compared with controls.

Table 8
Combinative toxicity of AFB_1 and FB_1 toxin in BEAS-2B

Concentration (IC_{50})	AFB_1 (μM)	FB_1 (μM)	Binary mixture (μM)	Viability (% control ± SD, $n = 6$)
0	0	0	0	100 ± 8
0.13	100	44.4	144.4	55 ± 9*
0.25	100	88.8	188.8	50 ± 8*
0.38	100	133.2	233.2	49 ± 6*
0.50	100	177.5	277.5	48 ± 5*
0.75	100	266.3	366.3	38 ± 7**
1.00	100	355.1	455.1	26 ± 6**

* $p < 0.05$ as compared with controls.
** $p < 0.01$ as compared with controls.

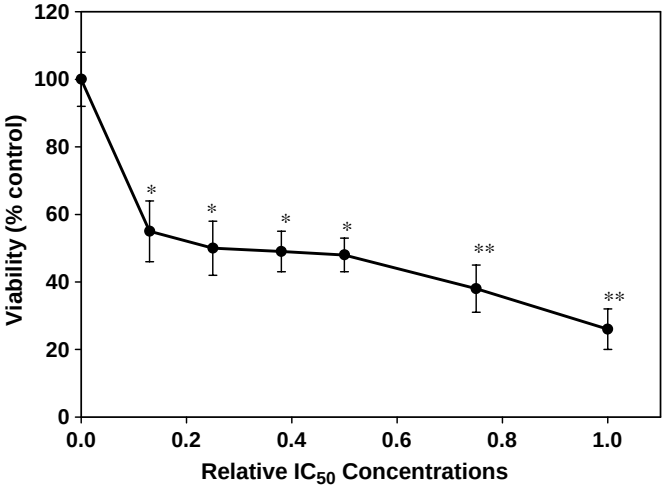

Fig. 2. Effect of AFB_1 and FB_1 on cellular viability in BEAS-2B cell line. Values represent mean viability as % control ± SD ($n = 6$). The IC_{50} was determined by probit analysis to be 196.9 μM. *$p < 0.05$ as compared with controls; **$p < 0.01$ as compared with controls.

Table 9
Summary of combinative toxicity of binary mycotoxin mixtures

Binary mixture	Estimated $LD_{50}/LC_{50}/IC_{50}$[a]	Measured $LD_{50}/LC_{50}/IC_{50}$[b]	Interaction index[c] (K)
F344 (mg/kg bw)	2.71	1.37	1.98
Mosq. (μg/l)	2661	1341.7	1.98
$HepG_2$ (μM)	200.1	333.0	0.60
BEAS-2B (μM)	277.5	196.9	1.41

[a] Estimated $LD_{50}/LC_{50}/IC_{50}$ determined by dose/concentration addition of 0.5 (each measured $LD_{50}/LC_{50}/IC_{50}$ of two toxins).
[b] Measured $LD_{50}/LC_{50}/IC_{50}$ determined by probit analysis.
[c] Interaction index is the ratio of estimated $LD_{50}/LC_{50}/IC_{50}$ divided by measured $LD_{50}/LC_{50}/IC_{50}$.

determined to be 0.60 and can be described as a weak antagonistic effect (Table 9).

Similar IC_{50} value of FB_1 at fractions of 1.0, 3/4, 1/2, 3/8, 1/4, 1/8, and 0 (control) was administered in combination with a constant concentration of AFB_1 (100 μM) to BEAS-2B cells. The results differ somewhat from those in $HepG_2$ or with the individual toxins. The cellular viability was affected greatly even in the lowest dose groups. The maximal effect occurred in the highest dose group equal to 26% of control values (Table 8, Fig. 2). The measured IC_{50} was 196.9 μM and the interaction index was calculated to be 1.41 demonstrating a strong additive interaction (Table 9).

4. Discussion

Previous studies have demonstrated not only the carcinogenic potential of AFB_1 in male F344 rats, but also the acute toxic response has been well quantified. Wong and Hsieh (1980) reported the oral LD_{50} in rat models as ranging from 5.5 to 17.9 mg/kg bw AFB_1, which is in relative agreement with our findings of 2.71 mg/kg bw when considering that our model employed more sensitive younger rats (≈120 g) than previous studies. Also differences could be derived from dietary factors between studies or methods of calculating the median lethal dose.

To our knowledge the acute toxic effects of AFB_1 in mosquitofish have never been tested; however, various investigations have demonstrated that the mechanism of action is highly conserved across species including those aquatic species tested (Gallagher and Eaton, 1995; Eaton and Groopman, 1994). Many of these models have demonstrated to be very sensitive including trout (p.o. LD_{50} 0.81 mg/kg bw) and zebra fish larvae (LC_{50} 0.51 mg/l) (Cullen and Newberne, 1994). Our results demonstrated that the mosquitofish model was sensitive to acute AFB_1 toxicosis with an LC_{50} value of 0.681 mg/l.

AFB_1 has been studied extensively in vitro and is reviewed by IARC (1993, 2002). Our study design included two cell lines: (1) the human hepatocellular carcinoma $HepG_2$ cells and (2) the human bronchiolar epithelial BEAS-2B cells. $HepG_2$ cells were employed because these cells maintained many functions associated with fully differentiated primary hepatocytes including Phase I and Phase II enzyme activities (Ehrlich et al., 2002; Tolleson et al., 1996). To our knowledge, the cytotoxic dose response of $HepG_2$ in regard to AFB_1 exposure has not been fully characterized; our results (IC_{50} 1.0 μM AFB_1) are in line with similar studies where IC_{50} values ranged from 0.065 μM in B-CMV1A2 cell line to 14 μM in BE12-6 cells (Lewis et al., 1999; Terse et al., 1993).

BEAS-2B cell is an SV-40 immortalized cell line originating from normal human bronchiolar epithelium (NHBE) progenitor cells (Reddel et al., 1988). It remains non-tumorigenic though numerous passages and represents a good model for studying pulmonary carcinogenesis because BEAS-2B originates from cells known to be targeted by chemical carcinogens in the lung (Reddel et al., 1988). We found that there was no cytotoxicity induced by AFB_1 at concentrations up to 100 μM, which is consistent with previous report (Macé et al., 1994). In a recent study, Van Vleet et al. (2002) measured the cytotoxicity of AFB_1 in normal Beas-2B cell line as well as those transfected with cDNA for CYP 1A2 or 3A4. Non-transfected BEAS-2B cells produced no metabolites and the IC_{50} was determined to be 348 μM by the MTT assay, which is in agreement with our results.

This study focused on testing acute combinative cytotoxicity of AFB_1 and FB_1 on these two human cell lines and used standard treatment procedure of 24 h. To address the question that results may be different if treating cells over a longer period of time, we carried out preliminary studies with extended treatment times (48 and 72 h) and found that no significant effect for up to 72 h treatment with same AFB_1 concentrations and FB_1 concentrations lower than 80 μM in these cells (data not shown).

The acute toxicity of FB_1 has rarely been addressed in vivo; FB_1 has generally characterized FB_1 as not acutely toxic through the oral route, while no studies have been published specifically targeting the lethality of single doses of pure FB_1 in laboratory animals (Bhandari et al., 2002). This is in agreement with our findings in that treatment of male F344 rats by gavage with $\leqslant$46.4 mg/kg bw did not result in mortality, however apparent toxic symptoms where noted (e.g., reduced feed intake and lameness). Low oral bioavailability of FB_1 most likely plays an important role in the lack toxicity in our model (Shephard et al., 1994).

With the relatively recent discovery of FB_1 (Gelderblom et al., 1988), and the finding of the inhibition of ceramide synthase (Wang et al., 1991) there have been few publications going beyond traditional toxicological studies involving laboratory rodents and common in vitro techniques. This study is the first to quantify the dose response of FB_1 in an aquatic model. Mechanistic studies in aquatic model have demonstrated that the initial action of FB_1 in aquatic species is similar to that reported in other species and seems to be a highly conserved mechanism (Goel et al., 1994; Meredith et al., 1998; Voss et al., 2001). It is thus expected that FB_1 would impact mosquitofish through

the same mechanism of inhibition of ceramide synthase initiating the cascade of events referred to previously. In this model we would expect that there would be a better dose response seen than in our other models, primarily since the method of administration allows for prolonged intimate contact between the organism and FB_1. Our results support this conclusion which demonstrates, by the calculated LC_{50} of 4.64 mg/l FB_1, that this model is relatively sensitive to the toxic effects of FB_1.

The toxic effects of FB_1 in vitro have been known for some time, however the specific mechanism of action resulting in the toxic response of FB_1 is very complex, possibly cell type specific, and not fully understood (Norred et al., 1991; Shier et al., 1991; Terse et al., 1993). In a recent review Gutleb et al. (2002) pointed out that tissue specificity in response to FB_1 induced cytotoxicity exists in a number of mammalian cell lines with IC_{50} concentrations ranging from 2.8 to 69.3 μM (Shier et al., 1991). With Lewis et al. (1999) reporting the IC_{50} of FB_1 in B-MCV neo cl 12 cells as 659 μM by the MTT assay FB_1 is generally not considered to be overtly toxic in the majority of cell lines.

Since the discovery of FB_1, in vitro techniques have served as the primary method to elucidate the mechanism by which this mycotoxin exerts its toxic effects. In an interesting study by Yoo et al. (1992) it was noted that the earliest effect of FB_1 in vitro is on sphingolipid metabolism. It was found that FB_1 inhibited proliferation and was cytotoxic to LLC-PK_1 (pig renal epithelial cells) with the effect on proliferation occurring at doses preceding cytotoxicity (10 and 35 μM versus >35 μM, respectively). The inhibition of ceramide synthase, however, was the first event in the cascade of events. This inhibition was also found to have a parallel dose response between the resulting increased Sa/So ratios and cytotoxicity only on a different time scale (Yoo et al., 1992). It was subsequently noted that there was also a concentration dependent decrease in more complex sphingolipids proposed to be one of the downstream impacts of ceramide synthase inhibition (Yoo et al., 1996).

The effect of combinative toxicity with binary toxins in F344 rats as shown in Table 5, a dose of AFB_1 (3/4 of the LD_{50}) in the presence of FB_1 (25 mg/kg) gave 100% mortality, was different from toxic effect observed in only AFB_1-treated rats, in which 20% mortality in 2.15 mg/kg AFB_1 group. These results suggest that a synergistic toxic effect occurred with the binary toxins, because FB_1 at 25 mg/kg resulted in no acute effects. The combinative toxic effect of this binary toxins, according to the model described by Tallarida (2001), was also estimated in the margin of synergistic effect ($K = 1.98$). Similar results were found in BEAS-2B (Table 8), in which a combination with FB_1 (44.4 μM) an approximately 50% reduction in viability was noticed, while the FB_1 concentration is about 8-fold below the IC_{50} value. It also suggested a synergistic cytotoxic effect from this single concentration of mixture. However, synergistic toxic effect or additive toxic effect for binary toxins in a model can not be determined by a single dose effect and should be determined by multiple concentrations simultaneously used in the model, including if dose–responsive relationships existed. As shown in Table 9, additive cytotoxic effect on BEAS-2B was concluded ($K = 1.41$).

Even though, to our knowledge, the cytotoxic dose response has not been replicated in either the $HepG_2$ or the BEAS-2B cells, parallels can be drawn and between our results and those of others. Neither cell line was determined to sensitive to FB_1 induced cytotoxicity (<200 μM) after 24 h incubation with a calculated IC_{50} of 399.2 μM in $HepG_2$ and 355.1 μM in BEAS-2B. This is common as far as the time lag between exposure and impacts manifested by this endpoint. As noted in LLC-PK1 cells, the decrease in complex sphingolipids does not become apparent until 24–48 h after cells have been exposed to FB_1 and before inhibition of cell growth or increased cell death at 48 h (Yoo et al., 1996).

The co-contamination of food stuffs with these two mycotoxins is well known and has been implicated in the development of human hepatocellular carcinoma in high risk areas around the world (Wang et al., 1995; Ueno et al., 1997), in addition to other well established risk factors, such as chronic infection with hepatitis B and C virus (IARC, 1994). There has been at least a partial characterization of these two mycotoxins effects individually; however, there have been few studies that have made an attempt to characterize their potential combined effects in any model. Food samples from endemic areas of primary liver cancer in China revealed an association with FB_1 as well as co-contamination with AFB_1 (Ueno et al., 1997). The resultant co-exposure has been suggested to be a risk factor for development of primary liver cancer (Ueno et al., 1997). Gelderblom et al. (2002) found when rats were treated in a sequential manner with AFB_1 and FB_1, there was a significantly increased cancer initiating potency. In addition to the cancer promoting activity of FB_1 on AFB_1 initiated hepatocytes, AFB_1 pre-treatment seemed to enhanced the FB_1 initiating potency, presumably by rendering the liver more susceptible to the toxic effects of FB_1 (Gelderblom et al., 2002). Studies of the co-contamination of AFB_1 and FB_1 in the same food sample on growing barrows and turkey poults resulted in additive to synergistic responses in the barrows and lass than additive to additive in the poults relative to individual exposures (Harvey et al., 1995; Kubena et al., 1995). The review of the immunobiological effects of co-exposure to AFB_1 and FB_1 resulted in different influences on the immune parameters relative to the individual toxins effects (Theumer et al., 2002) while a synergistic effect on reduction of body weight gain was observed due to oral administration of AFB_1 and FB_1 on male Wistar rats (Pozzi et al., 2001).

In conclusion, we found that mixtures of AFB_1 and FB_1 led to altered toxic effects compared to the individual compound administration with this combination being the most potent of all tested in our lab evidenced by the change in potency demonstrated by the interaction index. Increases

in the interaction index up to 1.98 were seen in two of the four models (Table 9). Only $HepG_2$ demonstrated weak antagonistic effect with this combination. It was found that at low doses there was an increase reported in the cell viability, however, as concentrations increased the impact on cellular viability did become apparent. This is difficult to interpret from a mechanistic point of view and is beyond the scope of this paper. For the most part, these results were expected since dual phase reactions are very common in the complex mixture studies, as demonstrated by decreased toxic effect in lower concentrations and increased toxic interactions at higher concentrations (Carpenter et al., 1998). It can be gathered that this binary mycotoxin mixture may pose a significant threat to public health and further research needs to be completed addressing alterations in toxin disposition that may influence the toxic manifestations in combination.

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