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Comparative acute and combinative toxicity of aflatoxin B_1 and T-2 toxin in animals and immortalized human cell lines

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ABSTRACT: Aflatoxin B_1 (AFB_1) and T-2 toxin (T-2) are important food-borne mycotoxins that have been implicated in human health and as potential biochemical weapons threats. In this study the acute and combinative toxicity of AFB_1 and T-2 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 to 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 T-2 to obtain LD_{50}, LC_{50} and IC_{50} values for the combination in each model. All models demonstrated a significant dose response in the observed parameters to treatment. The potency of the mixture was gauged through the determination of the interaction index metric. The results of this study demonstrate that these two toxins interacted to produce alterations in the toxic responses generally classifiable as additive; however, a synergistic interaction was noted in the case of BEAS-2B. 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. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: aflatoxin; T-2 toxin; biotoxin; mycotoxin; combinative toxicity; cytotoxicity

Introduction

Toxic fungal metabolites, mycotoxins, are structurally diverse compounds that represent the most important category of biologically produced 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, they 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 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 in the range 0.3–9.0 mg kg^{-1} body weight (bw). AFB_1 is also known to be 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 RNA, DNA and other protein constituents (Massey *et al*., 1995). 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).

Tricothecenes are a group of mycotoxins produced by *Fusarium* species. One of the most important tricothecenes is T-2 toxin, which is the common name for 4*beta*,15-diacetoxy-3*alpha*,dihydroxy-8*alpha*-[3-methylbutyryl-oxy]-12,13-epoxytrichothec-9-ene. T-2 toxin is

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the product of *F. sporotrichoides*, *F. poae*, *F. equiseti* and *F. acuminatum* and its production was usually enhanced by the unusual field conditions of prolonged wet and cold weather during harvest. There are many diverse mechanisms by which T-2 can produce toxicity and the relative importance of each in the production of the response is not fully understood (Coulombe, 1993). It is thought that inhibition of protein synthesis and its immunosuppressive properties are the most important human health impacts (Ueno, 1983, 1984; Yarom *et al*., 1984; Jagadeesan *et al*., 1982).

Co-exposure to multiple mycotoxins invokes cause for 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 to the 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 coexistence 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. This study extended research efforts to test the toxicity of the AFB_1 and T-2 combination in animals (F344 rats and mosquitofish) and human cells (BEAS-2B and HepG2).

Materials and Methods

Materials

The mycotoxins selected for this study AFB_1 and T-2 toxin were either purchased from Sigma Chemical Co. (St Louis, MO) or were kindly provided by various research units of U.S. Food and Drug Administration. The purity of each toxin was tested with high performance liquid chromatography for AFB_1 or gas chromatography for T-2. Stock solutions were made with dimethylsulfoxide (DMSO) and kept under argon. The 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.

Animals and Treatment

F344 Rats

Young male Fischer 344 rats (90–110 g) were obtained from Harlan Lab Animals Inc. (Indianapolis, IN) a week before experiments were initiated, and were housed individually in stainless steel cages under controlled temperature (22° ± 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^{-1} body weight, 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 5–7 groups. Each group included 6–12 animals. One group was given only solvent vehicle (DMSO) and used as the control. The other groups were administered by gavage various fractions of the derived LD_{50} for each mycotoxin, respectively. The study was conducted 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.

Mosquitofish

Mosquitofish (*Gambusia affinis*) (6 months old) were purchased from Ken's Hatchery & Fish Farm, Inc. (Alapaha, GA) or Carolina Biological Supply Co. (Burlington, NC) 3 weeks before experiments were performed. After arrival, the 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^{-1}), equipped with seasoned biological filters, and underwent quarantine procedures (treated once with trisulfa or every other day for 6 days with supersulfa). Goldfish flake fish food (Aquarium Pharmaceuticals, Inc., Chalfont, PA) was daily supplied *ad libitum*. After a week adaptation, the fish were further separated according to their gender into 40 l aquaria under similar conditions. Healthy fish (half male and half female) were randomly assigned into 3 l glass aquaria at the third week and were treated with individual toxins or combinations at various concentrations after 1 day adaptation. The treated and control fish

were observed for 5 days with morbidity and mortality recorded. The results were analysed and LC_{50} determinations made by probit analysis.

Human Cell Lines

$HepG_2$ 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^{-1}), hydrocortisone (0.5 μg ml^{-1}), insulin (5 μg ml^{-1}), bovine pituitary extract (35 μg ml^{-1}), ethanolamine (500 nM), phosphoethanolamine (500 nM), transferrine (10 μg ml^{-1}), 3,3′,5-triiodothyronine (6.5 ng ml^{-1}), epinephrine (500 ng ml^{-1}), retinoic acid (0.1 ng ml^{-1}) 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 the 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 in 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 *Fmax* microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 440 nm with background subtraction at 600 nm. The percent viability of the population of cells in each well was expressed as: OD of treated cells/OD of control cells × 100%.

Data Analysis

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 the percent viability of the population of cells in each well. The IC_{50} and 95% CI were also calculated by the SPSS 11.0 software using probit analysis. 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 was determined to be non-toxic through the dose range employed, the dose of that toxin was held constant over the regimen and is not included in the LD_{50} determination in that model (Tallarida, 2001).

Results

Acute Toxicity of AFB_1 and T-2 in F344 Rats

Doses of 1.0, 2.15, 4.64 and 10.0 mg kg^{-1} bw were used for AFB_1. As shown in Table 1, the higher doses of AFB_1 (10.0 and 4.64 mg kg^{-1} 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^{-1} bw AFB_1. Mortality reached 100% in animals treated with 4.64 mg kg^{-1} bw AFB_1 at

Table 1. Acute toxicity of AFB_1 or T-2 in F344 rats

Dose (mg kg^{-1} bw)	No. of animals	No. of AFB_1 deaths	AFB_1 mortality (%)	No. of T-2 deaths	T-2 mortality (%)
Control[a]	5	0	0	0	0
1.00	5	0	0	0	0
2.15	5	1	20	0	0
4.64	5	5	100	5	100
10.00	5	5	100	5	100

Time of observation was 7 days.
[a] Control animals received DMSO.

Table 2. Summary of LD_{50} determinations in F344 rats by Horn's method

Mycotoxin	LD_{50} (mg kg^{-1} bw)	95% CL (mg kg^{-1} bw)
AFB_1	2.71	2.00–3.69
T-2 toxin	3.71	2.69–5.01

96 h post-treatment. Twenty percent mortality was observed in animals treated with 2.15 mg kg^{-1} bw AFB_1 during the 1 week study period. No mortality was observed in animals treated with the lowest dose (1.0 mg kg^{-1} bw) or in the vehicle control group. The LD_{50} was determined to be 2.71 mg kg^{-1} AFB_1 (Table 2).

A similar dose range was used for testing acute toxicity of T-2 toxin; the results are shown in Table 1. The higher doses of T-2 toxin (10.0 and 4.64 mg kg^{-1} bw) resulted in acute toxic symptoms, such as refusal of food and diarrhea shortly (1–2 h) post-treatment. Within 24 h, 100% mortality (5/5) was observed in animals treated with 10 mg kg^{-1} bw and 4.64 mg kg^{-1} bw of T-2 toxin. In animals treated with 2.15 mg kg^{-1} bw T-2 toxin severe toxic symptoms appeared, such as refusal of food and bloody feces, however, no deaths were observed during the 1 week study period. No apparent toxic symptoms were observed in animals treated with the lowest dose (1.0 mg kg^{-1} bw) or in the vehicle control group. The LD_{50} was determined to be 3.71 mg kg^{-1} T-2 (Table 2).

Acute Toxicity of AFB_1 and T-2 in Mosquitofish

The higher concentrations of AFB_1 (2.15 and 1.0 mg l^{-1}) caused acute toxic symptoms post-treatment, such as reduction of activity and loss of righting reflex. Mortality appeared between 24–72 h in higher concentrations of AFB_1. Within 72 h, 100% mortality (12/12) was observed in fish treated with 1.0 and 2.15 mg l^{-1}. No mortality was observed in the two lowest concentrations of AFB_1-treated fish during 5 day study period (Table 3). The study yielded a LC_{50} value of 681 μg l^{-1} AFB_1 (Table 4).

Table 4. Summary of LC_{50} determinations in mosquitofish by probit analysis

Mycotoxin	LC_{50} (μg l^{-1})	95% CL (μg l^{-1})
AFB_1	681	420–800
T-2 toxin	147	95–227

The higher concentrations of T-2 (1.0 and 0.464 mg l^{-1}) caused acute toxic symptoms immediately after treatment. Mortality appeared within 2 h after treatment. Within 48 h, 100% mortality (12/12) was observed in fish treated with 1.0 mg l^{-1} of T-2. Within 72 h, 100% mortality was observed in fish treated with 0.464 mg l^{-1}. Deaths were observed after 72 h in the two lowest concentrations of T-2. Higher mortality of 75% (9/12) was found in fish treated with 0.25 mg l^{-1} and 33% (4/12) was found in fish treated with 0.1 mg l^{-1} during 5 day study period (Table 5). The study yielded a LC_{50} value of 147 μg l^{-1} T-2 (Table 4).

Acute Cytotoxicity of AFB_1 in $HepG_2$ and BEAS-2B Cells

The cytotoxic effects of AFB_1 at doses of 0.01, 0.1, 1, 10 and 100 μM in the human hepatoma cell line $HepG_2$ 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 (Table 5). Conversely, exposure of the human bronchiolar epithelial cell line BEAS-2B 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.

To test the cellular viability of $HepG_2$ and BEAS-2B in response to T-2 toxin at doses of 0.01, 0.1, 1, 10 and 100 μM T-2 was administered in each cell line. The results revealed that T-2 toxin has similar effects on cellular viability as AFB_1 in $HepG_2$ cells with an IC_{50} of 0.98 μM. More potent cytotoxic effects were observed in

Table 3. Acute toxicity of AFB_1 or T-2 in mosquitofish

Concentration (μg l^{-1})	No. of mosquitofish AFB_1/T-2	No. of AFB_1 deaths	AFB_1 mortality (%)	No. of T-2 deaths	T-2 mortality (%)
Control[a]	12/12	0	0	0	0
100	12/12	0	0	4	33
215	12/12	0	0	9	75
464	12/12	0	0	12	100
1000	12/12	12	100	12	100
2150	12/12	12	100	12	100

Time of observation was 5 days.
[a] Control animals received DMSO.

Table 5. Summary of IC_{50} determinations in $HepG_2$ and BEAS-2B cells by probit analysis

Cell line	AFB_1 IC_{50} (nM)	AFB_1 95% CL (nM)	T-2 IC_{50} (nM)	T-2 95% CL (nM)
$HepG_2$	1000	900–7440	980	0–1220
BEAS-2B	ND	ND	32.1	33.3–180

ND-not determined.

the BEAS-2B cell line treated with T-2, demonstrated by the calculated IC_{50} of 32.1 nM with the 95% confidence limits ranging from 3.28 nM to 179.86 nM (Table 5).

Toxic Effect of Binary Mycotoxin Mixtures in F344 Rats

Based on the determined LD_{50} values, experiments were carried out testing the acute combinative toxicity for these two toxins in each model following the outlined procedures. Symptoms of acute toxic effects, such as dizziness, bloody diarrhea and subcutaneous bleeding, appeared within a few hours post-treatment. Death of animals appeared within 24 h. As shown in Table 6, 100% mortality was observed in the high dose groups of 1.0 and 3/4 LD_{50}. Seventy percent mortality was observed in the animal group treated with 1/2 the calculated LD_{50} dose. Furthermore, 10% mortality was observed in the 3/8 and 1/4 LD_{50} treatment groups. No death was found in the group treated with 1/8 LD_{50} of each toxin. All surviving animals treated with 1/2, 3/8 and 1/4 LD_{50} doses had apparent toxic symptoms, such as yellowish skin, reduced body weight and ascites. Animals treated with 1/8 LD_{50} did not show significant toxic effects. The combinative LD_{50} for AFB_1 and T-2 was calculated as 2.83 mg kg^{-1} bw with 95% confidence limit at 2.41–3.37 mg kg^{-1} bw. The combinative toxicity index was found to be 1.144 (Table 10).

Toxic Effect of Binary Mycotoxin Mixtures in Mosquitofish

Experiments completed in the acute phase revealed that the mosquitofish was a sensitive fish model for our study following outlined procedures. The results of these experiments are shown in Table 7. The higher

Table 6. Combinative toxicity of AFB_1 and T-2 toxin in F344 rats

Dose (LD_{50})	AFB_1 (mg kg^{-1} bw)	T-2 toxin (mg kg^{-1} bw)	No. of animals	No. of deaths	Mortality (%)
Control[a]	0	0	10	0	0
1/8	0.34	0.46	10	0	0
1/4	0.68	0.93	10	1	10
3/8	1.02	1.39	10	1	10
1/2	1.36	1.86	10	7	70
3/4	2.03	2.79	10	10	100
1.0	2.71	3.71	10	10	100

Time of observation was 14 days.
[a] Control animals received DMSO.

Table 7. Combinative toxicity of AFB_1 and T-2 toxin in mosquitofish

Concentration (LC_{50})	AFB_1 (μg l^{-1})	T-2 toxin (μg l^{-1})	No. of fish	No. of deaths	Mortality (%)
Control[a]	0.00	0	12	0	0.00
1/8	85.1	18.4	12	2	17
1/4	170.3	36.8	12	3	25
3/8	255.4	55.1	12	8	67
1/2	340.5	73.5	12	10	83
3/4	510.9	110.4	12	12	100.0
1.0	681.0	147.0	12	12	100.0

Time of observation was 5 days.
[a] Control animals received DMSO.

Table 8. Combinative toxicity of AFB_1 and T-2 toxin in HepG2 cells

Concentration (IC_{50})	AFB_1 (nM)	T-2 (nM)	Binary mixture (nM)	Viability (% control ± SD)
0 (control)[a]	0	0	0.000	100 ± 6
0.13	125	123	248	69 ± 3
0.25	250	245	495	58 ± 1
0.38	375	368	743	57 ± 1
0.50	500	490	990	49 ± 1
0.75	750	735	1485	47 ± 1

Time of treatment was 24 h.
[a] Control cells received DMSO.

concentrations of these two toxins caused acute toxic symptoms, such as reduction of activity and loss of righting reflex. Mortality occurred within 24 h post-treatment and there were significant dose-dependent differences in mortality rates observed. Based on these data, the binary LC_{50} for AFB_1 and T-2 was calculated as 234.26 μg l^{-1} with 95% confidence limits at 178.65–291.11 μg l^{-1}. The combinative toxicity index ($K = LC_{50}$) estimated (414 μg l^{-1})/LC_{50} measured (234.26 μg l^{-1}) was found to be 1.77 demonstrating strong additive toxic effects of these two mycotoxins in this model (Table 10).

Cytotoxic Effect of Binary Mycotoxin Mixtures in Human Cell Lines

Concentrations of AFB_1 and T-2 toxin were equivalent to 3/4 (75%), 1/2 (50%), 3/8 (37.5%), 1/4 (25%), 1/8 (12.5%) and 0 (0%) of the individual IC_{50} values and administered for 24 h following the procedures outlined in the methods section. After exposures the viability of the each cell line was measured, and as presented in Table 8 and Fig. 1, the combined administration of AFB_1 and T-2 in HepG2 cells resulted in a good dose-response relationship with minimal cellular viability (46.25%) reported in the highest dose group and ranging up to 68.72% of control in the 1/8 (12.5%) group. The interaction index was determined to be 0.93 and as it approached unity it demonstrated the additive effect of these toxins in this hepatic cell (Table 10).

In BEAS-2B, there was no apparent cytotoxicity due to the administration of AFB_1 (<100 μM); however, T-2 toxin was demonstrated to be very cytotoxic to this cell line (IC_{50} = 32.1 nM). In order to examine the effects of these two mycotoxins, a broader dose range of 3/4 (75%), 1/2 (50%), 3/8 (37.5%), 1/4 (25%), 1/8 (12.5%), 1/16 (6.3%) and 0 (0%) of the IC_{50} values was applied, respectively (Table 9, Fig. 2). A good dose response was observed yielding a maximal effect on viability of 20.44% of control in the highest dose group. The effect on viability ranged from this level to a minimal effect of 61.57% of control. The interaction index was determined to be 9.69 and since this far exceeds unity, it demonstrates the synergistic interaction of AFB_1 and T-2 in this human lung cell line (Table 10).

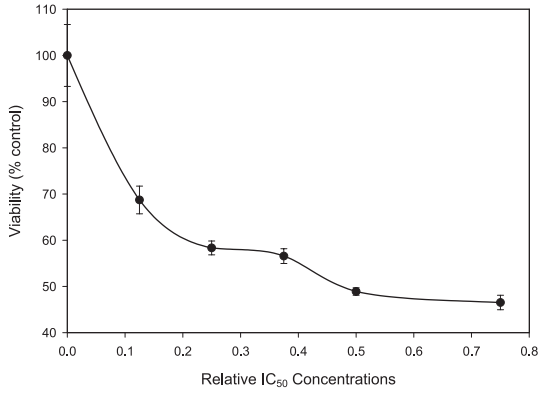

Figure 1. Effect of AFB_1 and T-2 on cellular viability in $HepG_2$ cell line. Cells were treated for 24 h. Values represent mean viability as % control ± SD (n = 6). The IC_{50} was determined by probit analysis to be 1.06 μM

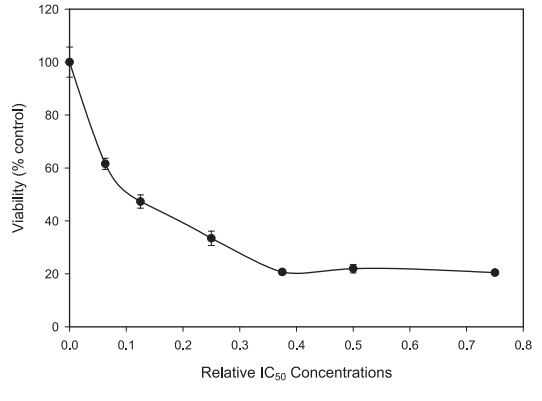

Figure 2. Effect of AFB_1 and T-2 on cellular viability in BEAS-2B cell line. Cells were treated for 24 h, values represent mean viability as % control ± SD (n = 6). The IC_{50} was determined by probit analysis to be 3 nM

Table 9. Combinative cytotoxicity of AFB_1 and T-2 toxin in BEAS-2B cells

Concentration (IC_{50})	AFB_1 (μM)	T-2 (nM)	Binary mixture (nM)	Viability (% control ± SD)
0 (control)[a]	0	0	0	100 ± 5
0.06	100	2	100,002	62 ± 2
0.13	100	4	100,004	47 ± 2
0.25	100	8	100,008	33 ± 2
0.38	100	12	100,012	21 ± 2
0.50	100	16	100,016	22 ± 2
0.75	100	24	100,024	20 ± 0

Time of treatment was 24 h.
[a] Control cells received DMSO.

Table 10. Summary of binary toxin mixtures

Binary mixture	Estimated LD_{50} or IC_{50}[a]	Measured LD_{50} or IC_{50}[b]	Interaction index[c] (*K*)
F344 (mg kg^{-1} bw)	3.22	2.83	1.13
Fish (μg l^{-1})	414	234.26	1.77
$HepG_2$ (μM)	0.99	1.06	0.93
BEAS-2B (nM)	32	3	9.69

[a] Estimated LD_{50} or IC_{50} determined by dose addition of 0.5 (each measured LD_{50} or IC_{50} of two toxins).
[b] Measured LD_{50} or IC_{50} determined by probit analysis.
[c] Interaction index (estimated LD_{50} or IC_{50}/measured LD_{50} or IC_{50}).

Discussion

In this study design AFB_1 and T-2 were employed because of the overwhelming wealth of scientific information that has been obtained over the past four decades and the recent revelations that both these toxins potentially have been weaponized (Christopher *et al*., 1997; Zilinskas, 1997). Previous studies have demonstrated not only the carcinogenic potential of AFB_1 in male F344 rats, but also the acute toxic response had been quantified as well. The oral LD_{50} in rat models ranged from 5.5 to 17.9 mg kg^{-1} bw AFB_1 (Busby and Wogan, 1984), which is in relative agreement with our findings of 2.71 mg kg^{-1} bw. It is expected that the present model would be more sensitive than that of the previous studies since the study employed what were predicted to be more sensitive younger rats (120 g) versus more developed mature male rats.

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 been demonstrated to be very sensitive to AFB_1, including trout (p.o. LD_{50} 0.81 mg kg^{-1} bw) and zebra fish larvae (LC_{50} 0.51 mg l^{-1}) (Cullen and Newberne, 1994). The present results demonstrated that the mosquitofish model was more sensitive to acute AFB_1 toxicosis with an LC_{50} value of 681 μg l^{-1}.

AFB_1 has been studied extensively *in vitro* and was reviewed by the International Agency for Research on Cancer (IARC, 1993). Our study design included two cell lines: (1) the human hepatocellular carcinoma cell line $HepG_2$ and (2) the human bronchiolar epithelial cell line BEAS-2B. The $HepG_2$ was employed because this cell line has 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). Although to our knowledge, the cytotoxic dose response of $HepG_2$ in regard to AFB_1 exposure has not been fully characterized, the present results (IC_{50} 1.0 μM AFB_1) are in line with similar studies in which the 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). The BEAS-2B cell line is an SV-40 immortalized cell line originating from normal human bronchiolar epithelium (NHBE) progenitor cells (Reddel *et al*., 1988). It remains non-tumorigenic through 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). In a recent study, Van Vleet *et al*. (2002) measured the cytotoxicity of AFB_1 in this cell line as well as those transfected with cDNA for CYP 1A2 or 3A4. Nontransfected BEAS-2B cells produced no metabolites of AFB_1 and the IC_{50} was determined to be 348 μM by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is in agreement with our results.

No cytotoxicity induced by AFB_1 was found at concentrations up to 100 μM.

The acute effects of T-2 toxin in the rat have been known for decades, and as a result this model has been used extensively. Various studies have yielded oral LD_{50} values ranging from 2.8 to 3.8 mg kg^{-1} bw, which is in excellent agreement with our findings of 3.71 mg kg^{-1} bw in F344 rats (for review see Hussein and Brasel, 2001). Toxic symptoms of acute T-2 toxicity in F344 rats, including lethargy, reduced food intake, hypertension and finally tachycardia followed by hypotension and death, were also reported by others (Wannemacher *et al*., 1991). No studies have been reported with T-2 in mosquitofish and as a matter of fact, we were not able to find any dose response data in aquatic models. The LC_{50} value of 147 μg l^{-1} obtained by this study proved this model to be the most sensitive bioindicator for either mycotoxin tested.

Under these conditions the $HepG_2$ cell line was less sensitive to T-2 induced cytotoxicity as evidenced by the IC_{50} value of 0.98 μM. Babich and Borenfreund (1991) reported an IC_{50} value of 21 nM which is much closer to the response obtained in BEAS-2B. Other cell lines have yielded IC_{50} values with T-2 ranging from 2 nM in the rat hepatoma cell line to 154.6 nM in human primary kidney cells (IARC, 1993; Babich and Borenfreund, 1991). The BEAS-2B cell line was a sensitive model in response to T-2 with an IC_{50} of 32 nM, which is in relatively good agreement with the reported value of 13 nM in the same cell line measured by the MTT assay (Lewis *et al*., 1999).

Currently little is known with regard to the co-occurrence and biological effect of a combination of AFB_1 and T-2. Based on the different optimum conditions for the production of each toxin, these two would not be predicted to coexist in the environment. However, the globalization of food markets affords the opportunity for co-exposure, and both toxins are known to have been potentially developed as biological weapons, hence justifying the study of combination effects from the public health, military and regulatory perspectives. Previous studies in male broiler chicks, male albino rats and Chinese hamsters have yielded results ranging from significant synergistic effects to less than additive effects on various endpoints (Huff *et al*., 1988; Tamimi *et al*., 1997; Rajmon *et al*., 2001). In this study this combination gave mixed results between models. Overall this combination could be described as additive in the mosquitofish, F344 rats and $HepG_2$ cells (Table 10). BEAS-2B cells, on the other hand, demonstrated the strongest response noted for any combination or model with the interaction index equal to 9.69. This is interesting since AFB_1 was not cytotoxic alone in this cell line; however, it potentiated the cytotoxicity of T-2 in combination. This may point to another mechanism of action in combination for AFB_1 because BEAS-2B has no metabolic activity toward it (Van Vleet *et al*., 2001).

In conclusion, it was found that mixtures of AFB_1 and T-2 led to altered toxic effects compared with the individual compound administration. This was evidenced by the change in potency demonstrated by the interaction index. The combination AFB_1 and T-2 in BEAS-2B resulted in the most potent interaction observed in this study with an interaction index of 9.69. The same combination in $HepG_2$ cells, however, resulted in additive effects. Overall this study demonstrated that these two toxins interacted to produce alterations in toxic responses. It can be deduced that these combinations may pose a significant threat to public health and further research needs to be conducted addressing alterations in toxin disposition that may influence the toxic manifestations in combination.

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