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Yvonne P. Dragan
*Ohio State University*, Yvonne.dragan@takeda.com

Wayne R. Bidlack
*California State Polytechnic University - Pomona*

Samuel M. Cohen
*University of Nebraska Medical Center*, scohen@unmc.edu

Thomas L. Goldsworthy
*Integrated Laboratory Systems, Inc*

Gordon C. Hard
*American Health Foundation*

*See next page for additional authors*

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# FORUM

# Implications of Apoptosis for Toxicity, Carcinogenicity, and Risk Assessment: Fumonisin $B_1$ as an Example

Yvonne P. Dragan,* Wayne R. Bidlack,† Samuel M. Cohen,‡,[1] Thomas L. Goldsworthy,§ Gordon C. Hard,¶ Paul C. Howard,‖ Ronald T. Riley,‖‖ and Kenneth A. Voss‖‖

*Ohio State University, James Cancer Center, 1148 James CHRI, 300 W. 10th Avenue, Columbus, Ohio 43210–1240; †California State Polytechnic University-Pomona, College of Agriculture, 3801 W. Temple Avenue, Pomona, California 91768; ‡University of Nebraska Medical Center, Department of Pathology and Microbiology, 983135 Nebraska Medical Center, Omaha, Nebraska 68198–3135; §Integrated Laboratory Systems, Inc., Health Science Division, P.O. Box 13501, Research Triangle Park, North Carolina 27709; ¶American Health Foundation, One Dana Road, Valhalla, New York 10595; ‖Division of Biochemical Toxicology, HFT-110, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Drive, Jefferson, Arkansas 72079–9502; and ‖‖USDA, Toxicology and Mycotoxin Research Unit, P.O. Box 5677, Athens, Georgia 30604–5677

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**The rates of cell proliferation and cell loss in conjunction with the differentiation status of a tissue are among the many factors contributing to carcinogenesis. Nongenotoxic (non-DNA reactive) chemicals may affect this balance by increasing proliferation through direct mitogenesis or through a regenerative response following loss of cells through cytotoxic (oncotic) or apoptotic necrosis. In a recent NTP study in Fischer rats and B6C3F$_1$ mice, the mycotoxin fumonisin B$_1$ caused renal carcinomas in male rats and liver cancer in female mice. In an earlier study in male BD-IX rats, fumonisin B$_1$ caused hepatic toxicity and hepatocellular carcinomas. An early effect of fumonisin B$_1$ exposure in these target organs is apoptosis. However, there is also some evidence of oncotic necrosis following fumonisin B$_1$ administration, especially in the liver. Induction of apoptosis may be a consequence of ceramide synthase inhibition and disruption of sphingolipid metabolism by fumonisin B$_1$. Fumonisin B$_1$ is not genotoxic in bacterial mutagenesis screens or in the rat liver unscheduled DNA-synthesis assay. Fumonisin B$_1$ may be the first example of an apparently nongenotoxic (non-DNA reactive) agent producing tumors through a mode of action involving apoptotic necrosis, atrophy, and consequent regeneration.**

*Key Words:* **apoptosis; fumonisin; mycotoxins; kidney cancer; liver cancer; regeneration; carcinogenesis.**

## Introduction

Fumonisins are ubiquitous contaminants of corn and other grain products produced by *Fusarium verticillioides* (synonymous with *Fusarium moniliforme*) and several other *Fusarium* species (Gelderblom *et al*., 1988; Marasas, 1996). There are at least 14 known fumonisins, of which fumonisin B$_1$ (FB$_1$) is the most plentiful. Its occurrence in corn varies seasonally and geographically. Levels of FB$_1$ in corn can range from undetectable (less than a few parts per billion) to as high as 150 parts per million (ppm; Shephard *et al*., 1996). In the United States, processed corn products usually contain less than 2 ppm FB$_1$ (Pohland, 1996).

Consumption of corn molded with *F. verticillioides* can lead to leukoencephalomalacia in horses, pulmonary edema in pigs, and liver and kidney toxicity in these and other species (Dutton, 1996; IPCS, 2000). Numerous mycotoxins have been identified in *F. verticillioides*-contaminated corn. During the past decade, FB$_1$ has been identified as the major *Fusarium* mycotoxin producing these toxicities (Dutton, 1996; Gelderblom *et al*., 1993), and it has been hypothesized as a risk factor for human esophageal cancer in certain regions of southern Africa and China. Early studies showed that inclusion of *F. verticillioides* in the diet of rats produced significant hepatotoxicity with necrosis, fatty degeneration, bile duct proliferation and eventually fibrosis (Jaskiewicz *et al*., 1987; Marasas *et al*., 1984; Wilson *et al*., 1985). Because of its ubiquitous presence in corn and potential for widespread exposure, the Food and Drug Administration recently evaluated FB$_1$ in chronic bioassays in rats and mice that were performed at the National Center for Toxicological Research through the National Toxicology Program (Howard *et al*., 2001a; NTP Technical Report, 2001). The results of the bioassay indicated that FB$_1$ was carcinogenic, producing significant incidences of kidney tumors in male rats and liver tumors in female mice. Previous bioassays using male rats performed in South Africa resulted in liver tumors (Gelderblom *et al*., 1991).

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[1] To whom correspondence should be addressed. E-mail: scohen@unmc.edu.

A major biological effect of $FB_1$ is the induction of apoptosis in various *in vitro* and *in vivo* model systems (Tolleson *et al*., 1996). Apoptosis is a specialized process of cell death that is part of normal organ development and tissue maintenance, but it can also occur in response to various environmental stimuli and can be indicative of toxicity. Numerous proteins and other cellular constituents have been identified that are involved in the apoptotic process and that regulate its occurrence. These include several sphingolipids and other lipids affected by exposure to $FB_1$ (Gelderblom *et al*., 2001; Merrill *et al*., 2001). To evaluate the role of apoptosis as a mode of action for carcinogenesis, with $FB_1$ as a case study, the International Life Sciences Institute (North American Branch) convened an expert working-group in February, 1999, with members chosen based on their expertise in carcinogenesis, toxicology, pathology, biochemistry, and cell biology. Additional discussions regarding the material took place by direct communication between the members of the working group. A summary of their deliberations is presented in this manuscript.

## Cell Death and Carcinogenesis

Cell death is a fundamental biological process (Kerr *et al*., 1972; Wyllie *et al*., 1980). Although several specific types of cell death have been identified, two major categories have been distinguished, apoptotic and oncotic necrosis. Unfortunately, the term necrosis has frequently been used in the literature synonymously with the process of oncotic necrosis, and the term apoptosis has been used for apoptotic necrosis. As described by Majno and Joris (1995), the fundamental distinction between these two processes is ultimately based on morphologic characterizations. A committee of the Society of Toxicologic Pathologists has recommended that necrosis be used as the overall term for cell death, and that specific processes of cell death be identified with the terms apoptotic and oncotic necrosis (Levin *et al*., 1999). Nevertheless, the terminology of apoptosis (apoptotic necrosis) as distinct from necrosis (oncotic necrosis) is deeply ingrained in the literature, and both terms are used in this report.

As described by Majno and Joris (1995), apoptotic necrosis is morphologically identified by cytoplasmic shrinkage and karyorrhexis, in contrast to oncotic necrosis, identified by cytoplasmic swelling and karyolysis. Either cell death process can occur in individual cells or in multiple cells in clusters. Apoptosis has frequently been referred to as programmed cell death, but in reality, both apoptotic and oncotic necrosis occur through identifiable molecular biological pathways. Similarly, some have distinguished apoptotic from oncotic necrosis based on the absence of an accompanying inflammatory reaction with apoptotic necrosis, whereas in fact, inflammation can accompany either form of necrosis.

Of fundamental importance in considering the biological effects of either type of necrosis is the tissue response. The cells that die can be lost and not replaced, leading to atrophy, or the tissue can regenerate new cells to replace those lost to cell death, commonly referred to as regeneration. Cell renewal systems exist in most tissues to replace cells that die. Cell death, whether apoptotic or oncotic, is a common effect of chemical toxicity and is most often accompanied by regeneration and usually associated with development of hyperplasia. Regardless of whether there is ultimately an increase in the number of cells, or merely a replenishment of the cells that died, there is an increase in the number of cell divisions in the affected tissue (Cohen, 1998). The rate of cell division is frequently increased compared to normal tissue, but this is not always the case.

Cancer is commonly accepted as being a consequence of multiple genetic alterations arising from inherited mutations in germ cells or as a consequence of mutations in somatic cells, resulting in altered growth (Knudson, 1971). Also, although DNA replication has incredible fidelity, it is not absolute. Thus, despite extensive and efficient cellular DNA repair mechanisms, every time DNA replicates, there is potential for a mistake to occur which can go unrepaired. These mistakes are rare, estimated to occur at the rate of approximately 1 error per $10^{10}$ nucleotides per DNA replication. If the error occurs in a gene involved in the carcinogenic process, then a step is taken toward the formation of cancer. Numerous endogenous processes frequently result in cellular DNA damage, including oxidation, deamination, exocyclic adduct formation, depurination, and others. Most of these are repaired, but when unrepaired, a mutational event can result. These mistakes occur on a regular basis endogenously, and there is the potential for DNA damage, even without exposure to an exogenously ingested, DNA adduct-generating agent.

Thus, an agent can increase the risk of carcinogenesis either by damaging DNA so that there are more mistakes each time DNA replicates, by increasing the number of DNA replications, or by a combination of both processes (Cohen and Ellwein, 1990; 1991; Moolgavkar and Knudson, 1981). If the agent, or a metabolite, forms a mutagenic adduct and causes DNA damage, it is commonly referred to as genotoxic (DNA reactive). Agents that increase carcinogenesis by increasing the number of DNA replications have been referred to by a variety of names, including non-genotoxic carcinogens.

Cell death and compensatory regeneration has become a well established mode of action for a variety of non-genotoxic chemical carcinogens targeting several tissues (Cohen, 1998). The cytotoxicity that is produced by these chemicals has been either demonstrated to be, or assumed to involve, oncotic necrosis. However, theoretically, apoptotic necrosis should also engender regeneration and have the potential for increasing the likelihood of cancer induction. Thus, on theoretical grounds, apoptotic necrosis with consequent regeneration should have the same potential to produce a carcinogenic process as oncotic necrosis plus regeneration. Those chemicals, which have been extensively studied and involve cytotoxicity via oncotic necrosis with consequent regeneration, have a

non-linear dose response with respect to carcinogenicity (Cohen, 1998). Although cytotoxicity theoretically implies a threshold response, such thresholds for non-genotoxic carcinogens continue to be the source of considerable controversy. Similar considerations are theoretically involved in interpreting the carcinogenic risk assessment of chemicals that have a mode of action involving apoptotic necrosis, rather than oncotic necrosis, with consequent regeneration.

In addition to apoptotic necrosis resulting in regeneration and increased proliferation, other mechanisms have been suggested whereby apoptosis may contribute to the carcinogenic process (Goldsworthy *et al*., 1996; Lowe and Lin, 2000; Manning and Patierno, 1996). Apoptosis of some cells could potentially result in development of a population of other cells that become resistant to apoptosis, which could accumulate heritable genetic changes during an increased life-span. Alternatively, an increased susceptibility to the signals for apoptosis in some cells, if accompanied by less efficient DNA repair in the remaining cells, could increase the cell population at risk. Apoptotic necrosis could also serve as an anti-carcinogenic mechanism by killing pre-neoplastic or neoplastic cells that develop. These different effects are not mutually exclusive, so that more than one could be affecting carcinogenesis.

Several cellular components, such as specific proteins, have been identified that can induce apoptosis (Brenner and Kroemer, 2000). In addition, it is clear that various sphingolipid metabolites are signaling molecules in pathways that regulate apoptosis and cell survival (Brenner and Kroemer, 2000; Merrill *et al*., 1997). Ceramide synthase is a key enzyme in sphingolipid metabolism and in the production of various sphingolipids involved in apoptosis. $FB_1$ has been identified as an inhibitor of this enzyme, leading to significant alterations in sphingolipid metabolism in both kidney and liver (Merrill *et al*., 1997; Wang *et al*., 1991). It was not surprising, therefore, that apoptosis was identified in various *in vitro* cellular systems exposed to $FB_1$, or that apoptosis was identified in the kidney and liver in rodents administered $FB_1$. Thus, a chain of events linking $FB_1$-induced ceramide synthase inhibition, disrupted sphingolipid metabolism, the induction of apoptosis, and the development of kidney and liver tumors in rodents can be theoretically drawn, raising the issue of the role of apoptosis as a mode of action in carcinogenesis.

### Fumonisin $B_1$ Carcinogenesis in Rodents

Marasas (1996) examined the apparent relationship between the consumption of moldy corn and high esophageal cancer rates in humans in certain districts in South Africa. *F. verticillioides* was identified as a possible cause of the cancer, and its toxicity and carcinogenicity were evaluated in rats. *F. verticillioides* (toxic strain MRC 826) corn culture material fed to rats over a lifetime produced primary liver cancer and esophageal basal cell hyperplasia (Marasas *et al*., 1984). Another feeding experiment again demonstrated the hepatotoxicity and hepatocarcinogenicity of *F. verticillioides* culture material (strain MRC 826) in rats (Jaskiewicz *et al*., 1987). In a series of short-term, initiation-promotion experiments in rats, Gelderblom *et al*. (1988) identified two new mycotoxins in the *F. verticillioides* (MRC 826) corn culture material which induced gamma-glutamyl transferase (GGT)-positive foci in rat liver and named them $FB_1$ and $B_2$. Other fumonisins and other mycotoxins in MRC 826 culture material have been identified including the highly mutagenic compound fusarin C (Gelderblom *et al*., 1984). However, culture material of a fungus (*F. verticillioides* MRC 1069) producing primarily fusarin C was not hepatocarcinogenic (Jaskiewicz *et al*., 1987).

An experiment involving administration of purified $FB_1$ (stated to be greater than 90 percent pure) fed at a dose of 50 ppm in the diet for 2 years to BD-IX male rats, resulted in hepatotoxicity and carcinogenicity (Gelderblom *et al*., 1991). Hepatotoxicity, regenerative nodules, and cholangiofibrosis were present by 6 months, with cirrhosis and hepatocellular carcinomas being present at 18–26 months. Ten of 15 rats that died or were killed between 18 and 26 months of the experiment had hepatocellular carcinoma in contrast to none in the controls.

The diet used in the above experiment (Gelderblom *et al*., 1991) was composed predominantly (75% by weight) of cornmeal. This diet was likely associated with marginal deficiencies of a number of vitamins, such as thiamin, riboflavin and vitamin E, and also contained low levels of vitamin $B_{12}$, folate, biotin, choline, and methionine when compared to a diet such as AIN-76. Some of these dietary alterations are known to be associated with hepatotoxicity and hepatocarcinogenicity in rats. In addition, the animals that were fed 50 ppm $FB_1$ gained considerably less weight during the entire course of the experiment (approximately 12 to 25 percent less weight gain compared to controls) indicating that the selected dose of 50 ppm exceeded guideline levels for a maximum tolerated dose (MTD). A repeat study using lower doses ($\leq$25 ppm in the diet) of $FB_1$ did not produce hepatocarcinogenicity (Gelderblom *et al*., 1997).

Utilizing the Solt and Farber (1976) hepatocyte selection model, Gelderblom *et al*. (1992) found little or no evidence of initiating activity for $FB_1$. They did find that administration of 1000 ppm of $FB_1$ in the diet increased the number and size of the GGT-positive foci in male F344 rats. However this dose was also associated with severe toxicity and growth retardation. A subsequent study (Gelderblom *et al*., 1996) evaluated $FB_1$ administered in the diet between 10 and 500 ppm for 21 days following administration of 200 mg of diethylnitrosamine (DEN). An increased number and area of GGT and glutathione *S*-transferase, placental form (GSTP) foci were observed at 250 and 500 ppm. The sphinganine:sphingosine ratios in the livers of non-hepatectomized rats were increased at $FB_1$ doses of 50 through 500 ppm. Another study (Gelderblom *et al*., 1996) examined zero to 500 ppm of $FB_1$ administered for 21 days followed by partial hepatectomy. The rats were sacrificed 24 h

**TABLE 1**
**Results of the 2-Year Carcinogenesis Bioassay Performed at the National Center for Toxicological Research (NCTR)**

| | | | | Liver | | Kidney | |
|---|---|---|---|---|---|---|---|
| Species | Sex | Dose (ppm) | Number | Adenomas | Carcinomas | Adenomas | Carcinomas |
| Rat | M | 0 | 48 | 0 | 0 | 0 | 0 |
| | | 5 | 40 | 2 | 1 | 0 | 0 |
| | | 15 | 48 | 2 | 1 | 0 | 0 |
| | | 50 | 48 | 0 | 0 | 2 | 7 |
| | | 150 | 48 | 1 | 0 | 5 | 10 |
| | F | 0 | 48 | 0 | 0 | 0 | 0 |
| | | 5 | 40 | 0 | 0 | 0 | 0 |
| | | 15 | 48 | 1 | 0 | 0 | 0 |
| | | 50 | 48 | 0 | 0 | 1 | 0 |
| | | 100 | 48 | 0 | 0 | 0 | 1 |
| Mouse | M | 0 | 47 | 9 | 4 | 0 | 0 |
| | | 5 | 47 | 7 | 3 | 0 | 0 |
| | | 15 | 48 | 7 | 4 | 0 | 0 |
| | | 80 | 48 | 6 | 3 | 0 | 0 |
| | | 150 | 48 | 8 | 2 | 0 | 0 |
| | F | 0 | 47 | 5 | 0 | 0 | 0 |
| | | 5 | 48 | 3 | 0 | 0 | 0 |
| | | 15 | 48 | 1 | 0 | 0 | 0 |
| | | 50 | 47 | 16 | 10 | 0 | 0 |
| | | 80 | 45 | 31 | 9 | 0 | 0 |

later. $^{3}$H-thymidine labeling index was decreased in partially hepatectomized rats.

The National Toxicology Program (NTP) at the National Center for Toxicological Research (NCTR) facilities in Jefferson, Arkansas, recently completed a 2-year bioassay in rats and mice on $FB_1$ (Howard *et al*., 2001a; NTP Technical Report, 2001). The chemical was characterized as having greater than 96 percent purity, and it was administered in NIH-31 diet, which was ascertained as having contamination with $FB_1$ less than 60 ppb. The chemical was administered in the diet at doses of 0, 5, 15, 50, or 150 ppm $FB_1$ to groups of 48 male rats, or 0, 5, 15, 50, or 100 ppm $FB_1$ to groups of 48 female rats for 105 weeks. Groups of 48 male and 48 female mice were fed NIH-31 diet containing 0, 5, 15, 80, or 150 ppm in the males, or 0, 5, 15, 50, or 80 ppm in the females. F344/N Nctr BR rats and B6C3F$_1$/Nctr BR (C57BL/6N $\times$ C3H/HeN MTV) mice were used from the NCTR breeding laboratory. The animals followed the standard NTP protocol for evaluation of carcinogenicity, and the doses were chosen based on 28- and 90-day dose range-finding studies. Additional animals were used for interim sacrifices for evaluation of cell proliferation, apoptosis, hematology, clinical chemistry, urinalysis, and tissue sphingolipid parameters.

The relevant tumor findings from the NTP bioassay are presented in Table 1. In rats, the only tumors induced were in the kidney, with significant incidences only in males. In mice, there were significantly increased incidences of liver tumors in females but not males. No renal lesions or tumors were seen in mice.

In female rats, a single renal adenoma was seen at 50 ppm and one carcinoma was detected at 100 ppm. These incidences were not statistically significant, but these are uncommon tumors in the rat. In male rats, the incidences of renal tumors were significantly increased at 50 and 150 ppm. Although some of these tumors were typical renal cell adenomas and carcinomas produced by a variety of other chemicals, several showed greater anaplasia than commonly seen in rat kidney neoplasms and one showed sarcomatoid differentiation. Metastases to the lungs were also common. In addition to the adenomas and carcinomas, focal atypical tubule hyperplasia (ATH), a precursor lesion of renal neoplasms, was also observed, more frequently in male rats than in females, with significant incidences at 50 and 150 ppm in males (Hard *et al*., 2001).

In mice, the incidence of hepatic tumors was not significantly increased in males, but adenomas and carcinomas were increased at 50 and 80 ppm doses in female mice. Spontaneous tumors occurred in the male mice at predicted rates in response to the approximately 20% caloric restriction that occurred.

In summary, $FB_1$ has been identified as carcinogenic in male rat kidney (NTP Technical Report, 2001) and liver (Gelderblom *et al*., 1991) and in female mouse liver (NTP Technical Report, 2001). The reasons for differences between the results of carcinogenicity tests in the 2 rat bioassays could be due to a variety of differences in the respective laboratories, including differences in rat strain (BD-IX vs. F344/N Nctr), diet, and purity of $FB_1$, amongst others. Regardless, the $FB_1$ doses that resulted in tumors in these rodent bioassays were comparable

(≥50 ppm). It is also apparent that a carcinogenic effect is detected only at doses ≥50 ppm in the female mouse and ≥50 ppm in the male rat. There is also a distinct difference between males and females in their response to $FB_1$. It is unclear if this is due to hormonal differences between sexes. The sex differences in the carcinogenic effects of $FB_1$ are similar to some other chemicals that are carcinogenic in rodents, such as methyl *tert*-butyl ether, unleaded gasoline, hexachloroethane, chlordane, and 1,1,2-trichloroethane (Moser *et al*., 1997). However, even for those chemicals, the exact mechanistic basis for this difference is unknown.

## DNA Reactivity of Fumonisin $B_1$

A limited number of *in vivo* and *in vitro* studies that examined the potential genotoxicity of $FB_1$ and other fumonisins have been reported (IARC, 1992; NTP Technical Report, 2001; IPCS, 2000). These include the following: the Ames assay in Salmonella strains TA97, TA98, TA100, and TA102, with or without an S9 activation system; UDS in rat hepatocytes and in rats orally administered $FB_1$ 12 h prior to sacrifice; $^{32}$P-postlabeling; *in vivo* and *in vitro* DNA repair assays; differential DNA repair in several *E. coli* strains; SOS chromotests in *E.coli* PQ27; transforming activity in BALB/C 3T3 A 31–1–1 mouse embryo cells *in vitro*; and the mouse micronucleus assay. Increased chromosomal aberrations have been observed occasionally. Overall the studies indicate that fumonisins are non-genotoxic and non-DNA reactive. DNA adducts derived directly from fumonisins have not been reported. Furthermore, the $FB_1$ molecule does not have functional groups that would suggest direct DNA reactivity.

There has been some limited evidence that $FB_1$ can produce DNA damage indirectly by producing cellular oxidative damage (Sahu *et al*., 1998; Yin *et al*., 1998). Increased lipid peroxidation was reported in the livers of rats fed $FB_1$, and in primary hepatocytes treated with $FB_1$ (Abel and Gelderblom, 1998), as evidenced by: increased thiobarbiturate acid reactive substances (TBARS); increased TBARS in cultured hepatocytes associated with cytotoxicity and protected by alpha-tocopherol. Increased lipid peroxidation and DNA strand breaks have also been induced by $FB_1$ in isolated rat liver nuclei (Sahu *et al*., 1998). However, the chemical structure of $FB_1$ does not suggest pro-oxidant activity. Some of the findings suggesting cellular oxidative damage may be due to toxicity rather than a direct cellular effect of the chemical. Also, a recent study involving the feeding of $FB_1$ at 250 ppm to rats (Lemmer *et al*., 1999) concluded that $FB_1$ appears to induce liver toxicity independently from effects on lipid peroxidation, although $FB_1$ did potentiate the effect of iron on lipid peroxidation.

The weight of evidence strongly suggests that $FB_1$ and other fumonisins do not produce DNA damage directly and are not DNA-reactive.

## Fumonisin $B_1$ Toxicity

Several studies demonstrated hepatotoxicity and nephrotoxicity following dietary administration of cultures of *F. verticillioides*. Gelderblom *et al*. (1988) showed that purified $FB_1$ was hepatotoxic to male BD-IX rats, but it was not until 1993 that purified $FB_1$ was demonstrated to be hepatotoxic and nephrotoxic in male and female Sprague-Dawley rats (Voss *et al*., 1993). Serum cholesterol and triglyceride concentrations were elevated and liver enzymes, including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities, were elevated, indicative of hepatotoxicity. Microscopic changes were observed in the liver, including apoptosis, at 150 ppm, but not at lower doses. A significant dose-related decrease in relative kidney weight and apoptosis were found at all 3 concentrations in males. There were no effects on liver or kidney weights in female rats, but apoptosis was seen at doses of ≥ 50 ppm. Hepatotoxicity and nephrotoxicity were closely correlated with elevated free-sphingoid bases in liver, kidney, serum and urine (Riley *et al*., 1994).

Ninety-day feeding studies have demonstrated that apoptosis of individual renal tubular cells was produced by $FB_1$ in male rats at doses of ≥9 ppm, and in female rats, apoptosis was produced only at the highest dose of 81 ppm (Bucci *et al*., 1998; NTP Technical Report, 2001; Voss *et al*., 1995). In a detailed review of the kidney histopathology in the NTP 2-year carcinogenicity bioassay, Dr. Gordon Hard observed that evidence of nephrotoxicity, including apoptosis and regeneration, was sustained for the full period of $FB_1$ exposure at the doses ≥50 ppm in male rats. The sequence of events in the NTP bioassay and related studies clearly demonstrated that administration of $FB_1$ to male F344/N Nctr rats produced significant amounts of apoptosis followed by active regeneration, hyperplasia, and ultimately, renal tumors in male rats at doses of 50 and 150 ppm. A similar but less pronounced effect was seen in female rats at 100 ppm.

Since statistically significant morphologic tumorigenic effects on the kidney appear to be restricted to the male rat, $FB_1$ binding to $\alpha_{2u}$-globulin was evaluated. No effect on $\alpha_{2u}$-globulin in the male rat kidney was identified by immunohistochemistry (Bucci *et al*., 1998; Howard *et al*., 2001a,b; NTP Technical Report, 2001). The morphologic alterations in rat kidney following $FB_1$ administration also are not those usually observed following administration of chemicals having an effect on $\alpha_{2u}$-globulin. In addition, although the renal effect was statistically significant in the male rat kidney, there were also hyperplastic and neoplastic changes in the kidney of a few female rats in the bioassay, but not at a statistically significant level (Howard *et al*., 2001a,b; NTP Technical Report, 2001).

In the rodent liver, the changes appear to be more complex. There is no question, given the elevated liver enzyme activities in the serum, that liver toxicity occurred in rodents. Morphologically there was evidence of apoptotic and oncotic necrosis and changes indicative of regeneration (Gelderblom *et al*.,

1991; NTP Technical Report, 2001; Voss *et al*., 1993). This has been reported in the studies from South Africa and those performed at NCTR. However, in the rat, the changes reported in the studies in South Africa were significantly more pronounced and extensive, and were accompanied by the eventual development of hepatocellular carcinomas and cholangiocarcinomas (Gelderblom *et al*., 1991). The acute liver toxicity reported in the South African experiments was evident as apoptotic (reported as single-cell necrosis) and oncotic necrosis, accompanied by increased hepatocellular proliferation (Gelderblom *et al*., 1991). There were marked changes in hepatic architecture and formation of regenerative nodules. These changes progressed to cholangiofibrosis, cirrhosis, and to rat hepatocellular tumor development (Gelderblom *et al*., 1991). In a subsequent experiment (Lemmer *et al*., 1999), these regenerative changes were evaluated in rats fed 250 ppm of $FB_1$ for 4 weeks. Histopathological findings included apoptosis, necrosis, oval cell proliferation, the appearance of hepatocytes staining positive for GST (pi), and the progressive development of fibrosis and regenerative nodules. Accompanying these changes were increased expression of $\alpha$-fetoprotein, hepatocyte growth factor (HGF), transforming growth factor-$\alpha$ (TGF-$\alpha$), and significantly increased levels of TGF-$\beta_1$ and c-myc. Increased cyclin D1 expression has also been shown to be increased (Ramljak *et al*., 2000).

In mice, $FB_1$ did not show evidence of hepatic toxicity at doses $\leq$27 ppm after 90 days of feeding (NTP Technical Report, 2001; Voss *et al*., 1995). However, there was evidence of centrilobular and occasional mid-zonal apoptosis and accompanying increased mitoses. These changes were mild and confined to females fed 81 ppm. Additional evidence of hepatotoxicity included elevation of serum enzymes. In the bioassay performed at NCTR (Howard *et al*., 2001a; NTP Technical Report, 2001), hepatocellular apoptosis was significantly increased at 2 years, at doses of 50 and 80 ppm $FB_1$ in female mice, but was not statistically significant in males at any dose. Hepatocellular hypertrophy was significantly increased at doses of 15, 80, and 150 ppm in the males, and at 50 and 80 ppm in the females at 2 years. There was an increased incidence of hepatocellular neoplasia in the females at 50 and 80 ppm but not in the males.

In both the rat kidney and liver, the subchronic microscopic lesions, organ weight differences, and sphinganine and sphingosine elevations were reversible 3 weeks after the return from $FB_1$-contaminated to control diet (Voss *et al*., 1998).

In summary, there is a relatively close correlation between induction of apoptosis in rat kidney, rat liver, and mouse liver and the induction of neoplasia in these organs in various studies, although there were significant differences between studies performed at different institutions. Apoptotic necrosis was accompanied by regeneration. In kidney, the necrosis appears to be entirely apoptotic, whereas in liver it appears to be a combination of apoptotic and oncotic necrosis. Regardless of the pathway by which necrosis was produced, regeneration followed.

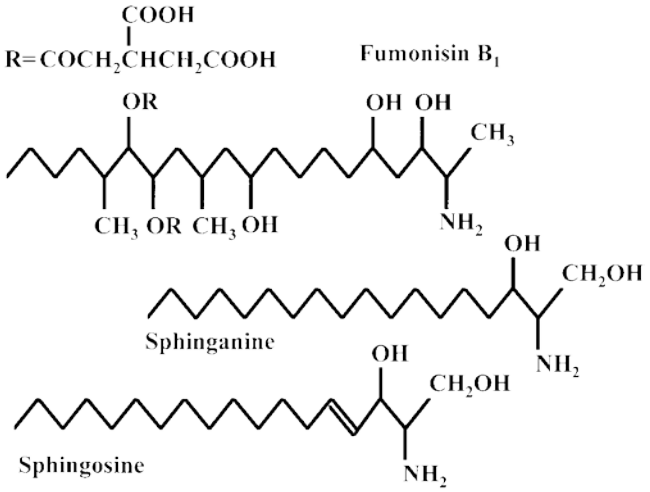


**FIG. 1.** Similarity in chemical structures of sphingosine, sphinganine, and $FB_1$.

$FB_1$ has a variety of toxic effects in other species in addition to rats and mice (NTP Technical Report, 2001). Hepatotoxicity and renal toxicity are common features in most species evaluated, including rabbits, horses, pigs, baboons, and vervet monkeys, after administration of either purified $FB_1$ or cultures of *F. verticillioides* (IPCS, 2000). In rabbits, pigs, and horses, the liver changes resemble those seen in rats and mice, including the presence of apoptosis. In the livers of baboons and vervet monkeys, hepatotoxicity was expressed as centrilobular necrosis with bile duct proliferation and fatty degeneration, occasionally with development of fibrosis. In addition, purified $FB_1$ has been identified as the cause of equine leukoencephalomalacia, apparently secondary to cardiovascular effects (Constable *et al*., 2000a). In pigs, cardiovascular effects appear to be expressed as pulmonary edema (Constable *et al*., 2000b; Smith *et al*., 2000).

### Fumonisin $B_1$ Effects on Sphingolipids

A common feature of exposure to $FB_1$ administration is perturbations in sphingolipid metabolism (Merrill *et al*., 1997; Riley *et al*., 1996; van der Westhuizen *et al*., 1999; Yoo *et al*., 1996). $FB_1$ is structurally similar to sphingoid bases (Fig. 1) and inhibits (Merrill *et al*., 1993; Wang *et al*., 1991) the enzyme ceramide synthase ($K_i$ ranges from 50 nM to 1 $\mu$M, depending on assay conditions) (Fig. 2). $FB_1$ binds to the catalytic site of ceramide synthase resulting in an inhibition of the condensation of sphinganine with fatty acyl CoA forming dihydroceramide. Furthermore, reacylation of sphingosine, formed from the breakdown of complex sphingolipids to form ceramide, is also inhibited by $FB_1$. As a result of the inhibition of ceramide synthase by $FB_1$ administration, the levels of free sphingoid bases are increased both in tissues, such as the liver

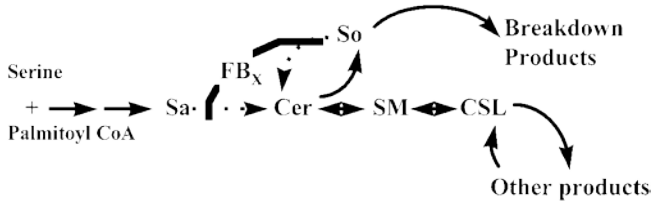


**FIG. 2.** Simplified schematic of *de novo* synthesis of ceramide and complex sphingolipids, sphingolipid turnover, and the location of the metabolic block (inhibition of ceramide synthase) imposed by $FB_1$. Biochemical consequences of this block are decreased *de novo* biosynthesis of ceramide and sphingosine, marked accumulation of sphinganine, accumulation of lesser amounts of sphingosine (formed by blocking the reutilization of sphingosine liberated during complex sphingolipid turnover), increased tissue ratio of sphinanine to sphingosine (a biomarker of exposure), increased levels of sphingoid base-1 phosphates and phosphatidylethanolamine, and a decrease in the *de novo* biosynthesis of complex sphingolipids.

and kidney, and also in the serum and urine (Merrill *et al*., 1993; 1997). This increase occurs at lower doses than those which produce apoptotic and/or oncotic necrotic effects or carcinogenicity (Riley *et al*., 1998). Increased sphingoid base concentrations have also been found in lung and heart, although apparently in the absence of apoptosis (Riley *et al*., 1993; Smith *et al*., 1999).

As a consequence of investigations on the effects of $FB_1$ on sphingolipid metabolism, there has been an explosion in research on the metabolism of these complex lipids and their relationship to the induction and inhibition of apoptosis (Merrill *et al*., 1993, 1997, 2001; Riley *et al*., 1998, 2001). Although the specific effects of sphingolipids on apoptosis are complex and not fully understood, there is no doubt that in various *in vivo* and *in vitro* cellular systems, administration of $FB_1$ produces alterations in sphingolipids with consequent alterations of apoptosis and cell proliferation. $FB_1$-induced elevation in free sphingoid bases induces apoptosis, whereas production of sphingosine-1-phosphate or inhibitors of ceramide biosynthesis can prevent apoptosis. Sphingosine-1-phosphate is also mitogenic in some cell systems. Effects on cell morphology and cell contact have been identified in response to alterations in sphingoid bases and more complex sphingolipids.

In addition to the effects of the disruption of sphingolipid metabolism on apoptosis and cell proliferation, $FB_1$ has been shown to alter other lipid pathways that also can affect apoptosis and cell proliferation (Merrill *et al*., 1997; 2001; Riley *et al*., 1998; 2001). Many of these effects on lipid metabolism could be a consequence of the effect of $FB_1$ on sphingolipid metabolism. Such effects include altered fatty acid and glycerophospholipid metabolism, altered expression of cytokines such as TNF-$\alpha$, alterations of expression of hormones or growth factors or their receptors, or direct or indirect effects on protein kinases, phospholipases, and cyclooxygenases. Effects on interactions of epithelial cells with the extracellular matrix, by inhibition of glycosphingolipids regulating cell recognition and adhesion, could also be a pathway leading to apoptosis.

Numerous regulatory pathways involving sphingolipids have been identified that can influence the apoptotic process (Merrill *et al*., 1997; 2001; Riley *et al*., 1998; 2001). Control of apoptosis is a complex, non-linear process that is cell specific and dependent on a variety of feedback mechanisms (Green and Reed, 1998; Brenner and Kroemer, 2000). Thus, disruption of sphingolipid metabolism and associated effects on glycerophospholipid and fatty acid metabolism could lead to multiple alterations in pathways controlling cell proliferation and cell death.

In Sprague-Dawley and Fischer 344 rats, New Zealand white rabbits, and BALB/c and other mouse strains, disruption of sphingolipid metabolism in liver and kidney occurs at $FB_1$ doses below those that produce morphologic evidence of injury (Voss *et al*., 2000). When liver pathology is observed, there is a close correlation between the incidence and severity of the pathology and the increase in free sphinganine, indicative of disrupted sphingolipid metabolism (Delongchamp and Young, in press; Riley *et al*., 1994). In rat kidney, sphinganine concentrations increase rapidly and are seen at doses not producing morphological evidence of toxicity. Urinary sphinganine levels also rise and are closely correlated with the severity of nephrotoxicity as determined by microscopic tissue examination (Howard *et al*., 2001b; NTP Technical Report, 2001; Riley *et al*., 1994).

## Fumonisin $B_1$ Toxicokinetics

$FB_1$ is a highly hydrophilic chemical, which is poorly absorbed from the gastrointestinal tract in all animal species examined (Dutton, 1996; IPCS, 2000). It is excreted largely intact in the feces, and this occurs rapidly (Shephard *et al*., 1992, 1994). In addition, in studies in rats following gavage administration (Shephard *et al*., 1994), little (approximately 0.2%) is excreted in the bile, indicating that there is minimal enterohepatic circulation. Following gavage administration, trace amounts were detected in urine, liver, kidney, and in red blood cells, and none was detected in plasma, heart, or brain. $FB_1$ does not appear to cross the placenta (Voss *et al*., 1996), and there is little evidence that it crosses the blood brain barrier.

There have also been investigations of the kinetics of $FB_1$ following administration by either the intraperitoneal or intravenous routes (Norred *et al*., 1993; Shephard *et al*., 1992, 1994). However, since $FB_1$ exposure in humans is via oral ingestion, these studies involving systemic administration may not be relevant to usual human exposures. Nevertheless, following systemic administration, approximately 60% of $FB_1$ is excreted in bile within four h.

$FB_1$ does not appear to accumulate in the tissues, but low levels appear to persist in the kidney and liver in the rat (Norred at al., 1993). The effects of $FB_1$ on accumulation of free sphingoid bases and toxicity are reversible (Voss *et al*., 1998; Wang *et al*., 1999), although the elevation of free sphin-

goid bases is persistent in kidney for several days after cessation of exposure to $FB_1$ (Wang *et al*., 1999). $FB_1$ metabolites have not been identified; most of the administered $FB_1$ can be accounted for as the unmetabolized chemical (NTP Technical Report, 2001).

In other mammalian species, including the non-human primate (vervet monkey), swine, cattle, and horses, the kinetics appear to be similar to those in the rat and indicate a low gastrointestinal absorption, rapid clearance, biliary excretion of absorbed $FB_1$, and accumulation of minor amounts of the administered dose in liver and kidneys (Dutton, 1996; IPCS, 2000; NTP Technical Report, 2001). Non-human primate studies (Shephard *et al*., 1994) produced evidence that gut microflora are capable of removing one or both tricarballylic acid groups from the molecule, but no evidence for hepatic metabolism has been reported. There is a paucity of data on the physiological and pharmacokinetic fate of $FB_1$ under chronic administration conditions.

## Fumonisin $B_1$ and Esophageal Cancer

The effects of fumonisins on humans have been difficult to determine, although chronic ingestion of moldy corn has been statistically associated with increased incidences of esophageal cancer in the Transkei region of southern Africa (van Rensburg, 1981; Rheeder *et al*., 1992; Marasas *et al*., 1988). In the Transkei region, corn is the mainstay of the diet and accounts for a majority of the calories ingested by this population. Marasas *et al*. (1981) noted that the proportion of kernels infected with *F. verticillioides*, the most prevalent fungi in maize in the Transkei, was significantly correlated with esophageal cancer rates. The regions with high exposure to moldy corn directly correlated with esophageal cancer incidence relative to low-exposure areas. The level of $FB_1$ was also higher in the high esophageal-cancer area compared to other parts of the world. Consumption of *F. verticillioides*-contaminated maize has also been associated with human esophageal cancer in China (Chang *et al*., 1992; Chu and Li, 1994).

However, many concerns regarding the epidemiology of the relationship between increased $FB_1$ consumption and the development of esophageal cancer have been identified (Craddock, 1992). For example, $FB_1$ is but one of many contaminant toxins from *Fusarium* that are present in corn, and there are other species of fungi also present (Shephard *et al*., 1996). In addition, contamination with other known carcinogenic compounds, such as nitrosamines and polycyclic aromatic hydrocarbons, are also high in these same regions (Burrell *et al*., 1966; Craddock, 1992; Lu *et al*., 1986). Importantly, several nutritional deficiencies have also been identified in these populations that could contribute to the high esophageal cancer rates, particularly in the high esophageal-incidence areas of South Africa (van Rensburg *et al*., 1985). These include not only specific vitamin deficiencies such as vitamins A and C and riboflavin, but importantly, there appears to be a zinc deficiency as well (van Rensburg *et al*., 1986). A study in 11 male baboons not administered $FB_1$ demonstrated that when riboflavin was omitted from their diet, esophageal lesions appeared, including hyperplasia with numerous mitotic figures (Foy and Kondi, 1984). Zinc deficiency has been identified as a critical factor in esophageal carcinogenesis in both human epidemiologic investigations and in animal models (Craddock, 1992; Fong *et al*., 1998; Newberne *et al*., 1997).

An additional confounding factor is the practice in the high-incidence areas of the Transkei to brew beer with contaminated corn resulting in extremely high levels of fumonisin (Marasas, 1995). Alcohol itself is a known risk factor for esophageal cancer, as are the high levels of nitrosamine contamination that can be present in some of these alcoholic beverages (Anderson *et al*., 1996; Newberne *et al*., 1997; Rogers *et al*., 1995; Siglin *et al*., 1995).

Increased incidences of esophageal cancer have also been identified in areas in northeastern Italy, where the consumption of corn has been associated with these neoplasms (Franceschi *et al*., 1990). However, these populations also appear to have markedly increased exposure to alcohol and tobacco, known risk factors for esophageal cancer. The effect of maize consumption on the increased incidence of esophageal cancer in Italy was significantly elevated only in those individuals who consumed excessive levels of alcohol and might have also been related to dietary insufficiencies, such as niacin and riboflavin.

Studies in animals have not clarified the relationship of $FB_1$ to esophageal cancer. Culture material from *F. verticillioides*, strain MRC 826, significantly enhanced nitrosamine-induced esophageal carcinoma in BD-IX rats (van Rensburg *et al*., 1982). However, when this rat strain was co-administered purified $FB_1$ with nitrosamine, there was no effect of $FB_1$ on esophageal cancer incidence (Wild *et al*., 1997). In separate animals, purified $FB_1$ was administered as 5 mg per kg by gavage for 4 weeks; there was an increase in the sphinganine/sphingosine ratio in the kidney and a slight increase in the liver, but there was no statistical increase in this ratio in esophageal tissue.

Overall, the combination of epidemiologic and animal studies provides some evidence that exposure to corn highly contaminated with *F. verticillioides* might be related to esophageal cancer; however, $FB_1$ itself does not appear to be an esophageal carcinogen.

## Implication for Risk Assessment

Corn consumption results in exposure to fumonisins, although the risk to human health from fumonisin exposure is difficult to determine (Shephard *et al*., 1996). The cardiovascular effects in horses and pigs may have relevance to human toxicity; however, the rather unique toxic responses to $FB_1$ in these species provide little insight as to potential carcinogenic risk for humans. Cardiovascular toxicity in rodents has not been fully evaluated.

Liver and kidney toxicity secondary to $FB_1$ administration appears to be a relatively common response across species, not only in rats and mice, but in rabbits, horses, pigs, and non-human primates (NTP Technical Report, 2001; Voss *et al*., 2000). Such toxicity, however, appears to occur at relatively high doses, usually measured in hundreds of micrograms of $FB_1$ per kilogram body weight per day. Human exposure in the U.S. and Europe is generally at levels of micrograms or less per kilogram per day. In high consumption areas such as the Transkei in southern Africa, the consumption of $FB_1$ can be much higher, approaching hundreds of micrograms per kilogram per day.

There are now three chronic bioassays with purified $FB_1$ which have produced a carcinogenic effect in rodents. An estimated no observed effect level (NOEL) for renal tumors is between 0.9 and 3.0 mg/kg/day for $FB_1$, and for female mouse liver tumors an estimated NOEL is 2.2–7.5 mg/kg/day based on lifetime exposure (NTP Technical Report, 2001; Howard *et al*., 2001a).

As indicated in the proposed cancer risk-assessment guidelines of the U.S. Environmental Protection Agency, mechanistic information should be included in any risk assessment when available. The possibility of DNA reactivity (genotoxicity) is of paramount importance. Based on the non-genotoxicity of $FB_1$ and its apparent mode of action involving toxicity and regeneration, a non-linear extrapolation to low doses and to humans is appropriate. Toxicity to the liver appears to be the most consistent end point across species, but toxicity (as evidenced by apoptosis) to the kidney appears to be slightly more sensitive, at least in the male rat. Although the toxic effects appear to be non-linear, the question as to whether these represent true biological thresholds or not remains unclear. There have been no biological effects that we are aware of demonstrated *in vivo* at a dose of less than 1 ppm in any species, except for erratic effects on the growth of castrated male pigs (Rotter *et al*., 1996). The growth effects were clearly present at 1 ppm. Overt toxicity in kidney is associated with quite high levels of sphinganine. However, it is unclear how disruption in sphingolipid metabolism in the various tissues leads to toxicity and what aspects of altered lipid metabolism contribute to the carcinogenic response. There continues to be a need for more detailed understanding of the effects of $FB_1$ on lipid metabolism, particularly with respect to its effects on apoptotic and oncotic necrosis in specific tissues. For example, what specific alterations in lipid metabolism lead to the induction of apoptosis and/or oncotic necrosis? What is the quantitative relationship between the preneoplastic and neoplastic effects?

Kuiper-Goodman *et al*. (1996) have reviewed various toxic endpoints and conducted a risk assessment based on these observations. Based on a sampling of food products and dietary patterns, they estimated an average intake of $FB_1$ of $<0.089$ $\mu$g/kg body weight for 5–11-year-old children and less in adults, well below levels estimated to pose a health risk. The conservative assumptions on the dietary intake of $FB_1$ have led to the assessment that the level of $FB_1$ in Canadian diets is between 1100 and 42,000 times lower than the no-observed-adverse-effect level (NOAEL) determined for $FB_1$ in rodents, horses, pigs, and monkeys. Similar estimates for exposure were observed for the general population in the Netherlands (de Nijs *et al*., 1998) and in Scandinavian countries (Nordic Council of Ministers, 1998). Individuals requiring a gluten-free diet, including patients with celiac disease or sprue, were identified as being at increased risk because of increased corn consumption. Nevertheless, even those individuals are estimated to consume considerably less than 1 $\mu$g/kg body weight per day, well below consumption levels in the highly contaminated regions of southern Africa (Marasas *et al*., 1997). More recently, Kodell *et al*. (2001) provided a risk assessment based on liver tumorigenesis in the female mouse (NTP Technical Report, 2001), utilizing a biologically based model that included parameters for cell deaths and cell births. Similar to the actual results in the 2-year bioassay, this model concluded that tumors would occur only at the highest doses of $FB_1$ administered, supporting a non-linear, margin of exposure approach to extrapolation of this data for human risk assessment.

Several gaps in our knowledge of the effects of $FB_1$ in rodents and in humans limit our ability to precisely extrapolate from animal models to humans. In addition, the human epidemiology data relating $FB_1$ exposure to esophageal cancer has several confounding factors, including a lack of detailed exposure analysis in some studies and the confounding of numerous other potential contributors to the carcinogenic process.

There are also several gaps in our knowledge with respect to research in animals and our understanding of the cell biologic effects of $FB_1$ treatment. Although extensive research has detailed the effects of $FB_1$ on sphingolipid metabolism, the precise alterations that occur in various tissues in different species, including humans, remain to be clarified. In particular, the critical alterations leading to the induction of apoptotic or oncotic necrosis in different cell types is yet to be delineated, and the relationship of these alterations to dose and other environmental factors, especially nutritional, are yet to be defined. More quantitative information over several time periods, and the effects of different doses of $FB_1$ on apoptotic and oncotic necrosis and regeneration are required. Lastly, the renal tumors produced in the male rat kidney are highly unusual, the sarcomatoid variant being a unique response so far described in the rat kidney. Is there an effect of $FB_1$ on differentiation processes in the rat kidney that could produce such an unusual response? Might other $FB_1$-induced effects work in concert to ultimately affect carcinogenesis?

## Summary

In summary, on theoretical grounds, apoptotic necrosis may be considered similar to oncotic necrosis with respect to a mode of action for risk assessment. That is, apoptosis leading

to a regenerative process involving sustained cell proliferation could, over time, lead to an increased incidence of tumors in the target tissue. Apoptotic necrosis and regeneration as a mode of action appears to be supported by the example of $FB_1$-induced renal tumors. However, the relationship of apoptosis to cancer development in response to $FB_1$ in the liver remains less clear, where apoptotic and oncotic necrosis apparently both occur. Nevertheless, in the bioassays performed so far, the target tissues clearly have regeneration consequent on apoptotic and/or oncotic necrosis present at the doses at which tumors were observed. These mechanistic findings, coupled with the lack of genotoxicity, provide a plausible mode of action for $FB_1$ carcinogenic effects at relatively high exposure levels and support a non-linear dose-response relationship.

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