Journal of Cellular Biochemistry 91:243–249 (2004)

Hyperplasia, Partial Hepatectomy, and the Carcinogenicity of Aflatoxin B_1

B.D. Roebuck*

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755

Abstract Generalized cellular hyperplasia has long been associated as a factor in the causation of liver cancer. Parenchymal cell hyperplasia resulting from hepatotoxins, viruses, parasites, or malnutrition is exceedingly variable as to when it occurs, its extent, and its duration. Partial hepatectomy has been used as an experimental tool precisely because the timing and extent of hyperplasia can be known and controlled. With regards to aflatoxin B_1 (AFB_1) carcinogenesis, partial hepatectomy has produced variable results. An explanation appears to reside in the hepatotoxic properties of AFB_1 that enhance the early stages of carcinogenesis. J. Cell. Biochem. 91: 243–249, 2004. © 2003 Wiley-Liss, Inc.

Key words: aflatoxin B_1; liver cancer; hepatectomy; hyperplasia; hepatic toxicity

HISTORICAL PERSPECTIVE

The aflatoxins including aflatoxin B_1 (AFB_1) are secondary metabolites of some strains of the molds *Aspergillus flavus* and *A. parasiticus* and related species that grow on food and feed crops [Wilson and Payne, 1994]. They are widely distributed in agricultural products such as peanuts and corn. Aflatoxins cannot be eliminated completely from the human food supply and represent a health concern for populations that cannot properly store agricultural commodities to limit mold growth or who have limited access to a wide variety of other foods. AFB_1 either alone or synergistically interacting with hepatitis B virus has long been associated with the causation of liver cancer [Kensler et al., 2003; Kew, in press]. Under conditions of exposure to aflatoxin-contaminated foods, it would be surprising if exposure to other hepatotoxic chemicals also did not occur. With worldwide deaths due to liver cancer of nearly one million [Chen et al., 1997] and a rising incidence of liver cancer in some developed countries (e.g., United States [El-Serag and Mason, 1999; Kim et al., 2002]), investigations

This manuscript was written in memory of Prof. Edward Bresnick. I first met Ed in 1986 when we both served on an advisory committee of a program project focused on hepatic carcinogenesis and cancer chemoprevention. Life flows in mysterious ways and in 1989, Ed moved from The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska to Dartmouth Medical School, to become Chairman of the Department of Pharmacology and Toxicology. Ed was my Chairman from 1989 to 1994 and he was a efficient and thoughtful administrator. Ed brought people together. One memorable example was his "recruitment" of cancer epidemiologists, molecular biologists, clinicians, and basic scientists for an intense series of seminars with the explicit goal of investigating the role of dietary calcium on the prevention of colonic cancer. In a short period of 3 months, we focused on an important cancer prevention issue, identified a funding source, and submitted a grant that was funded. Although the projects and players have changed, more than a decade later this collaboration remains as the Epidemiology and Cancer Chemoprevention Group within the Norris Cotton Cancer Center. Ed had very broad interests not only in cancer research, but also science in general. He firmly believed that science should serve humanity. Ed had a very special talent for knowing every departmental member on a professional as well as personal level. Frequently, Ed would wander into my office or laboratory to inquire of my work, my undergraduate teaching at Dartmouth College, or share something from his work. He always had positive words. Sharing a good story or joke, we always departed with a good laugh . . . and oh, yes . . . the mandatory "Get back to work!" as he headed down the hall. Thank you, Ed.

Grant sponsor: NIH; Grant number: CA 39416.

*Correspondence to: B.D. Roebuck, PhD, Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755, USA.
E-mail: Bill.D.Roebuck@Dartmouth.edu

Received 22 September 2003; Accepted 24 September 2003
DOI 10.1002/jcb.10758

must go beyond single chemical or infectious agents. Studies of interactions between agents at all stages of the long carcinogenesis process, especially during the early events of initiation and promotion, must be undertaken. Cellular proliferation and regeneration have a long association with cancer, but with regards to the aflatoxins, these processes have received too little attention.

PARTIAL HEPATECTOMY: CELLULAR EVENTS

An important model for studying cellular regeneration has been a partial hepatectomy. Higgins and Anderson [1931] characterized this model, which now has been used in hundreds of studies regarding the control and consequences of cellular regeneration [Michalopoulos and DeFrances, 1997]. Partial hepatectomy is a simple surgical procedure in which one or more hepatic lobes are removed intact without damage to the remaining lobes. Unlike treatment with hepatotoxins, such as carbon tetrachloride, partial hepatectomy is not associated with tissue injury and inflammation. The time of removal or "damage" is precisely known. The residual lobes grow first by hyperplasia and then by hypertrophy to match the mass of the lobes that were removed. All of the existing mature cell types composing the liver regenerate and the first to respond are the parenchymal cells. Nearly all the parenchymal cells of the remaining liver lobes participate in one or two proliferative cycles. Within 7–10 days, the liver has restored its mass to the original mass prior to partial hepatectomy.

CONFLICTING RESULTS REGARDING PARTIAL HEPATECTOMY AND AFB_1

Absence of an Effect

In the early 1970s, the first extensive examination of the effect of partial hepatectomy on AFB_1 carcinogenesis was undertaken [Rogers et al., 1971]. One might dismiss this article based upon its title, "Absence of an effect of partial hepatectomy on AFB_1 carcinogenesis," but an analysis of the data of their four experiments reveals much. In the first experiment, the effects of AFB_1 on cell proliferation were examined. A single dose of 100 μg AFB_1 per rat (approximately 0.5 mg/kg body weight) was given at the time of a two-thirds partial hepatectomy or 14 h later and proliferation was measured by both ^{3}H-thymidine labeling of nuclei as assessed by autoradiography and the counting of parenchymal cell mitoses in stained sections. Classic periportal necrosis was seen in all rats that were given AFB_1 when hepatectomy was performed and less extensive necrosis was observed in livers of rats treated 14 h following partial hepatectomy. At both time points, AFB_1 completely blocked cellular proliferation. And additionally, when AFB_1 was given at 30 h following partial hepatectomy and the liver was examined at 48 h relative to hepatectomy, there was also significant inhibition of the proliferation of parenchymal cells and no obvious necrosis.

In the second experiment, the effects of when AFB_1 exposure commenced following partial hepatectomy were examined. Daily doses of AFB_1 began at 6 h, 24 h, 48 h, or 6 days subsequent to a two-thirds partial hepatectomy and the cancer incidence was examined after 15 months. For each of these four times at which chronic AFB_1 treatment commenced, three different daily dose schedules were used such that the totals for these divided doses were 50, 100, or 375 μg AFB_1 per rat. Again, the AFB_1 was toxic and approximately 20% of the rats of each group died in the first 2 weeks of AFB_1 treatment. A 71% incidence of hepatocellular carcinoma was observed in the rats that received 375 μg AFB_1; whereas, the rats that received less AFB_1 had a significantly smaller cancer incidence. The incidence of hepatocellular carcinomas was not influenced by the time after hepatectomy at which AFB_1 commenced.

The third experiment had the same basic design as the second experiment; however, only a one-third partial hepatectomy was performed. And as in Experiment 2, one group began daily carcinogen treatments at 6 h and a second group began at 6 days post-hepatectomy. Both groups received a total dose of 375 μg AFB_1 over approximately a month of daily dosing. Compared to similar groups in Experiment 2 above, the one-third hepatectomy increased the cancer incidence, but not statistically. Furthermore, the cancer incidence did not differ with respect to whether AFB_1 treatment commenced at 6 h or 6 days following hepatectomy.

In the last experiment, rats received daily doses of 25 μg AFB_1 for a total dose of 125, 250, or 375 μg AFB_1. Three months following the initial dose of AFB_1, a two-thirds partial hepatectomy

TABLE I. Enhancement of Focal Growth by One-Third Partial Hepatectomy in an AFB_1-Induced Model of Hepatic Carcinogenesis*

	25 μg × 5 doses			25 μg × 10 doses		
	Control	Hepatectomy	% Change	Control	Hepatectomy	% Change
N	7	6		7	5	
Volume %	0.09 ± 0.02[a]	0.40 ± 0.16	445	0.46 ± 0.09	2.17 ± 0.56[b]	475
Diameter (μm)	124 ± 10	170 ± 21	137	152 ± 10	324 ± 45[b]	213
No./cm^3	452 ± 96	485 ± 106	7	954 ± 148	375 ± 55[b]	−60

*These data are from Davis [1995].
[a]The values are given as mean ± SE.
[b]Statistically different from control by *t*-test ($P < 0.05$).

was performed. At least 10 weeks elapsed between the last AFB_1 dose and the partial hepatectomy. There were more hepatocellular carcinomas in the rats that received the partial hepatectomy, but the incidence was not statistically significant.

Rogers et al. [1971] concluded that "hyperplasia per se is not an enhancing factor for AFB_1" and that "the induction of hyperplasia by AFB_1 itself is important in tumor development." They then implied that generalized hepatic hyperplasia was required. These conclusions were prophetic.

Enhancement of Carcinogenesis

As predicted [Rogers et al., 1971], partial hepatectomy can enhance AFB_1-induced hepatocarcinogenesis. Davis [1995] used a well characterized and quantitative, short-term model of liver cancer that had been used extensively in studies of AFB_1-induced hepatic carcinogenesis [Appleton and Campbell, 1982; Kensler et al., 1986, 1987, 1992; Roebuck et al., 1991; Bolton et al., 1993; Maxuitenko et al., 1993]. Briefly the model is as follows: AFB_1 (25 μg per rat) is given by gavage 5 days per week followed in 2 days by a second 5-day series of AFB_1, putative preneoplastic clusters of aberrant parenchymal cells (henceforth termed, foci) are identified in paraffin tissue sections by histochemical or immunohistochemical reactions. The foci are quantified at the light microscopic level by morphometric techniques [Pugh et al., 1983]. For AFB_1, it is recognized that the number, size, and volume percent of liver occupied by the foci is predictive of the ultimate development of cancer. Conceptually, the volume percent is equivalent to the hepatic tumor burden. The advantages of using this short-term model are that studies can be completed in 2–4 months and much fewer rats are required to yield quantitative results.

In Table I, the data clearly show that by two slightly different treatment protocols one-third partial hepatectomy enhanced AFB_1-induced hepatic carcinogenesis [Davis, 1995]. In the first experiment, rats were gavaged daily with 25 μg AFB_1 (0.25 mg/kg body weight) per day for 5 successive days and on the fifth day a one-third partial hepatectomy was performed. The second experiment was a variation of this theme with the addition of a second week of AFB_1 exposure that followed the partial hepatectomy. In both cases, 11 weeks after the initial exposure to AFB_1, stained sections of liver tissue were examined by light microscopy for foci. When partial hepatectomy was performed on the fifth day of AFB_1 treatment, the focal burden was increased from 0.09% to 0.40% of the liver volume. This 445% increase in the focal burden was essentially the result of an increase in the size of the foci since the number of foci did not change. An even greater increase (475%) in focal burden occurred in the group that received the second week of AFB_1 treatment that followed the partial hepatectomy. And again, this burden was the result of an increase in the size of the foci. In fact, the number of foci present in the liver was greatly reduced. The reason for a reduction in focal density is not known, but it could be related to the toxicity of the AFB_1 such that minimally transformed clusters of hepatocytes that having not acquired resistance to the toxic effects of AFB_1 are killed. The implications are that the first doses of AFB_1 initiated the foci (i.e., damage to DNA leading to a mutated hepatocyte) and subsequent doses promoted or selected for the growth of hepatocytes that were capable of growing in the toxic environment of AFB_1.

TOXICITY OF AFLATOXIN TO LIVER CELLS

AFB_1-Induced Cell Turnover

The aflatoxins including AFB_1 were discovered because of their hepatotoxicity to poultry, fowl, and livestock. The toxicity of AFB_1 has been extensively described and characterized [Newberne and Butler, 1969; Busby and Wogan, 1985]. Liu et al. [1988] quantified the killing of liver cells in the AFB_1 treatment regimen that was ultimately used by Davis [1995] and which is described in the previous section. The procedure of Yager and Potter [1975] was used to quantitatively measure the liver cell loss due to AFB_1 treatment. Young adult rats were subjected to a two-thirds partial hepatectomy and their hepatic DNA was labeled with ^{3}H-thymidine in doses of 20 μCi each at 22, 24, 26 h and 46, 48, 50 h following partial hepatectomy. These times corresponded to the two major peaks of DNA synthesis in the regenerating liver and with this procedure approximately half of the parenchymal cell nuclei were labeled. The carcinogenic regimen began 2 weeks following the prelabeling of the hepatic DNA. Periodically throughout the carcinogenic regimen, whole livers were removed for analysis of total ^{3}H-thymidine. The loss of prelabeled ^{3}H-thymidine from the hepatic DNA is an index of the hepatocyte loss from the liver [Yager and Potter, 1975]. The results of three separate experiments are illustrated in Figure 1. Rats did not grow during exposure to AFB_1 and some lost body weight (Fig. 1A). Similar observations of growth inhibition have been made and once the aflatoxin treatment ended, the rats regained the lost weight [Roebuck et al., in press]. Over the 2 week treatment period the livers of rats not treated with AFB_1 increased 20%; whereas, there was a loss of 10–45% of the liver weight compared to the initial liver weight for the aflatoxin-treated rats (Fig. 1B). In rats not treated with AFB_1, there was no loss of labeled DNA; whereas, in animals treated with AFB_1, there was a dramatic loss of ^{3}H-thymidine from prelabeled DNA (Fig. 1C). This cell loss ranged between 40 and 70% of the activity prior to AFB_1 treatment. Histological sections of liver revealed that even a single dose of 25 μg AFB_1 (0.25 mg/kg body weight) produced slight coagulative necrosis, hydropic changes, and pyknotic nuclei, which was largely limited to the periportal hepatocytes. This large hepatocyte turnover seemed surprising for such a small dose of AFB_1 and a short protocol [Liu et al., 1988] and certainly it appears comparable to a one-third to two-thirds partial hepatectomy. It should be emphasized that the treatment protocol used by Davis [1995], Liu et al. [1988], and others is a regimen that results in cancers in 1–2 years [Roebuck et al., 1991].

Solt–Farber Protocol

Solt and Farber [1976] developed a functional assay to examine the early events of chemical carcinogenesis (Fig. 2A). Their protocol evolved from the considerable evidence that cancer gradually arises from an altered population of cells and that most hepatocarcinogens inhibit liver cell division and are also generally

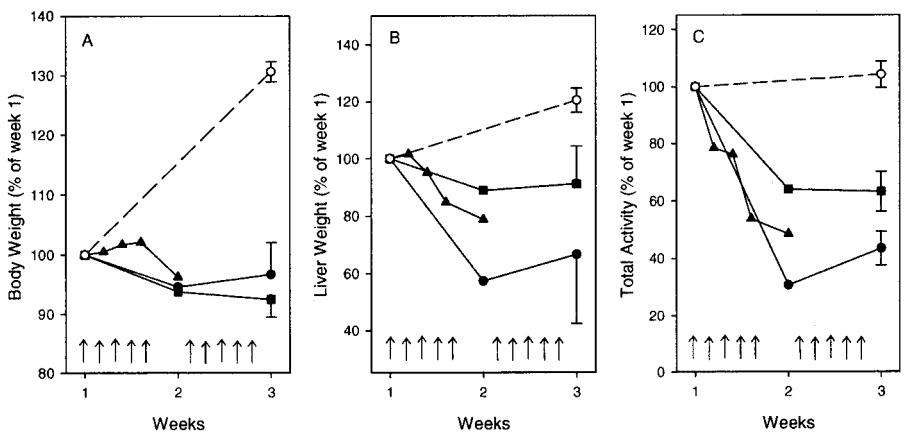

Fig. 1. Effects of a short-term AFB_1 treatment protocol on the liver. **A**: Body weight gain as affected by multiple doses of AFB_1 (arrows correspond to a daily dose of 25 μg per rat) for 5 days per week. Open symbols represent control rats that receive vehicle and closed symbols are for rats that received AFB_1. Three separate experiments with AFB_1 were undertaken and each replicate experiment is indicated by a different "filled symbol." Each symbol is the mean of four to six rats and representative standard error bars are shown. **B**: Liver weight gain of the same rats. **C**: ^{3}H-thymidine activity in the liver of the same rats.

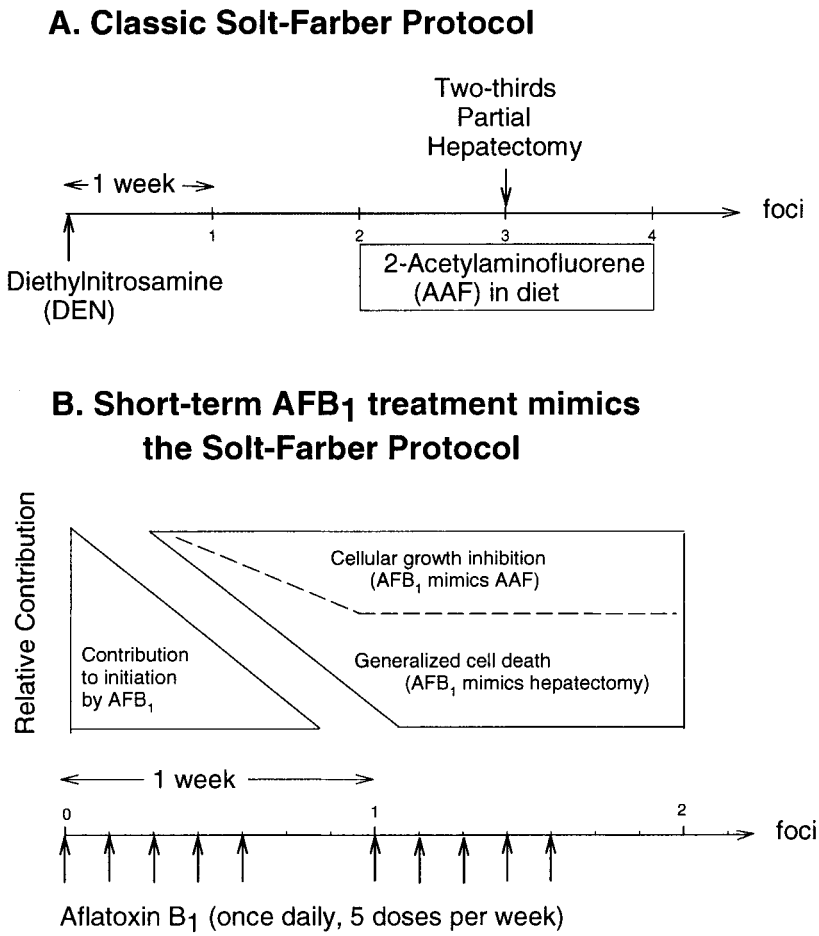

Fig. 2. Short-term AFB_1 exposure and the Solt–Farber protocol. **A**. Classic Solt–Farber protocol as redrawn from Solt and Farber [1976]. **B**: Short-term AFB_1 protocol with hypothesized relative contribution of initiation, growth inhibition, and cytotoxicity.

hepatotoxic. There are three components to their assay: Cancer is initiated by a single dose of carcinogen, for example, diethylnitrosamine, but numerous other hepatocarcinogens can be used including AFB_1. Two weeks later the rats are fed a growth inhibitor, such as 2-acetylaminofluorene, that suppresses the growth of the liver. The inhibitor is fed for 2 weeks. And finally, in the middle of feeding of the general growth-suppressing agent, a strong and generalized growth stimulus is applied to the liver. Typically this stimulus has been a two-thirds partial hepatectomy; however, hepatotoxic chemicals such as carbon tetrachloride have been used to stimulate liver growth [Cayama et al., 1978]. Microscopically identifiable foci are detectible at 30 h following partial hepatectomy and grossly visible nodules of aberrant cells are observed in 7–10 days. Functionally, the assay depends upon the selective growth of cells that are resistant to the cytotoxic effects of the growth inhibitor coupled with a local environment "demanding" proliferation of liver cells.

IMPLICATIONS OF HEPATOTOXICITY FOR AFB_1 CARCINOGENESIS

From the pioneering work of Rogers et al. [1971] with the insights afforded by the development of the Solt–Farber protocol, Davis [1995] showed that partial hepatectomy would promote or enhance AFB_1 carcinogenesis in a treatment regimen that by itself results in cancers [Roebuck et al., 1991].

To extend this work, it seems reasonable to hypothesize that AFB_1 not only initiates the carcinogenesis process, but also participates in its promotion by selection of the early putative preneoplastic foci. One can readily imagine that the Solt–Farber protocol (Fig. 2A) might be redrawn (Fig. 2B) for the aflatoxin treatment regimen used by Davis [1995]. Perhaps the first

dose of AFB_1 mostly damages DNA (i.e., initiates) and then in each succeeding dose, the AFB_1 assume two other roles. It inhibits hepatic cell division, which we know AFB_1 can do, and it kills parenchymal cells, which we know aflatoxin can also do. If this concept is correct, AFB_1 might be considered as "auto-promoting." With a carcinogenic dose of AFB_1 (i.e., 10 doses each of 0.25 mg per kg rat), we do not know what fraction of this dose is minimally needed for initiation and what fraction is required as a general hepatic growth inhibitor versus a lethal parenchymal cell toxin.

Aflatoxins and in particular AFB_1 are recognized human carcinogens, but the coupled role of toxin in the carcinogenic process is not well established. There are circumstances in which ascribing a cytotoxic or genotoxic role to an environmental exposure of AFB_1 would be useful. A synergistic interaction between hepatitis B virus infection and AFB_1 exposure has long been considered, but the mechanism of the interaction including a possible hepatotoxic or growth inhibitory role of AFB_1 has not been well characterized. An intriguing literature exists in which small, non-injurious exposures to the bacterial endotoxin, lipopolysaccharide, augments the toxicity of AFB_1 to produce significant damage to the liver of a rat [Barton et al., 2000]. Similar synergistic interactions have been shown for the classic plant hepatotoxin monocrotaline and lipopolysaccharides [Yee et al., 2003]. These agents and other hepatotoxins as well as infective agents and inflammatory processes may interact with AFB_1 in a manner similar to the Solt–Farber protocol to cause liver cancer.

ACKNOWLEDGMENTS

Dr. Davis completed her doctoral thesis in the laboratory of Dr. Thomas W. Kensler. The histopathological analyses of the liver sections were accomplished in the laboratory of Dr. Roebuck. We thank Dr. Kensler, Linda Conrad, and Karen J. Baumgartner for critical help in preparing the manuscript.

REFERENCES

Appleton BS, Campbell TC. 1982. Inhibition of aflatoxin-initiated preneoplastic liver lesions by low dietary protein. Nutr Cancer 3:200–206.

Barton CC, Ganey PE, Roth RA. 2000. Lipopolysaccharide augments aflatoxin B_1-induced liver injury through neutrophil-dependent and -independent mechanisms. Toxicol Sci 58:208–215.

Bolton MG, Muñoz A, Jacobson LP, Groopman JD, Maxuitenko YY, Roebuck BD, Kenlser TW. 1993. Transient intervention with oltipraz against aflatoxin-induced hepatic tumorigenesis. Cancer Res 53:3499–3404.

Busby WF, Wogan GN. 1985. Aflatoxins. In: Searle CE, editor. Chemical carcinogens. vol. 2. Washington, DC: American Chemical Society. pp 946–1093.

Cayama E, Tsuda H, Sarma DSR, Farber E. 1978. Initiation of chemical carcinogenesis requires cell proliferation. Nature 275:60–62.

Chen CJ, Yu MW, Liaw YF. 1997. Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroenterol Hepatol 12:S294–S308.

Davis EF. 1995. The protective role of oltipraz in multistage hepatocarcinogensis in the aflatoxin B_1 treated rat. Ph.D. Thesis, Johns Hopkins University, Baltimore, MD.

El-Serag HB, Mason AC. 1999. Rising incidence of hepatocellular carcinoma in the United States. New Engl J Med 340:745–750.

Higgins GM, Anderson RM. 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 12:186–202.

Kensler TW, Egner PA, Davidson NE, Roebuck BD, Pikul A, Groopman JD. 1986. Modulation of aflatoxin metabolism, aflatoxin-N^7-guanine formation, and hepatic tumorigenesis in rats fed ethoxyquin: Role of induction of glutathione S-transferases. Cancer Res 46:3924–3931.

Kensler TW, Egner PA, Dolan PM, Groopman JD, Roebuck BD. 1987. Mechanism of protection against aflatoxin tumorigenicity in rats fed 5-(2-pyrazinyl)-4-methyl-1,2-diothiol-3-thione (oltipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-ones. Cancer Res 47:4271–4277.

Kensler TW, Groopman JD, Eaton DL, Curphey TJ, Roebuck BD. 1992. Potent inhibition of aflatoxin-induced hepatic tumorigenesis by the monofunctional enzyme inducer 1,2-dithiole-3-thione. Carcinogenesis 13:95–100.

Kensler TW, Qian GS, Chen JG, Groopman JD. 2003. Translational strategies for cancer prevention in liver. Nat Rev Cancer 3:321–329.

Kew MC. Synergistic interaction between aflatoxin B_1 and hepatitis B virus in hepatocarcinogenesis. Liver Int (in press).

Kim WR, Brown RS, Terrault NA, El-Serag H. 2002. Burden of liver disease in the United States: Summary of a workshop. Hepatology 36:227–242.

Liu YL, Roebuck BD, Yager JD, Groopman JD, Kensler TW. 1988. Protection by 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) against the hepatotoxicity of aflatoxin B_1 in the rat. Toxicol Appl Pharmacol 93:442–451.

Maxuitenko YY, MacMillan DL, Kensler TW, Roebuck BD. 1993. Evaluation of the post-initiation effects of oltipraz on aflatoxin B_1-induced hepatic tumorigenesis. Carcinogenesis 14:2423–2425.

Michalopoulos GK, DeFrances MC. 1997. Liver regeneration. Science 276:60–66.

Newberne PM, Butler WH. 1969. Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A review. Cancer Res 29:236–250.

Pugh TD, King JH, Koen H, Nychka D, Chover J, Wahba G, He Y, Goldfarb S. 1983. Reliable stereological method

for estimating the number of microscopic hepatocellular foci from their transections. Cancer Res 43: 1261–1268.

Roebuck BD, Curphey TJ, Li Y, Baumgartner KJ, Bodreddigari S, Yan J, Gange SJ, Kensler TW, Sutter TR. Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. Carcinogenesis (in press).

Roebuck BD, Liu YL, Rogers AE, Groopman JD, Kensler TW. 1991. Protection against aflatoxin B_1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz): Predictive role for short-term molecular dosimetry. Cancer Res 51:5501–5506.

Rogers AE, Kula N, Newberne PM. 1971. Absence of an effect of partial hepatectomy on aflatoxin B_1 carcinogenesis. Cancer Res 31:491–495.

Solt D, Farber E. 1976. New principle for the analysis of chemical carcinogenesis. Nature 263:701–703.

Wilson DM, Payne GA. 1994. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In: Eaton DL, Groopman JD, editors. The toxicology of aflatoxins: Human health, veterinary, and agricultural significance. Boston: Academic Press, Inc. pp 309–325.

Yager JD, Potter VR. 1975. A comparison of the effects of 3′-methyl-4-dimethylaminoazobenzene, 2-methyl-4-dimethylaminoazobenzene, and 2-acetylaminofluorene on rat liver DNA stability and new synthesis. Cancer Res 35:125–1234.

Yee SB, Harkema JR, Ganey PE, Roth RA. 2003. The coagulation system contributes to synergistic liver injury from exposure to monocrotaline and bacterial lipopolysaccharide. Toxicol Sci 74:457–469.