Influence of Vitamins A, C, and E and \( \beta \)-Carotene on Aflatoxin B\(_1\) Binding to DNA in Woodchuck Hepatocytes

Ming-Whei Yu, Ph.D.,* Yu-Jing Zhang, M.D.,* William S. Blaner, Ph.D.,† and Regina M. Santella, Ph.D.*

Background. There is extensive epidemiologic evidence suggesting a protective role for micronutrients in cancer incidence. This evidence comes from studies of fruit and vegetable intake and serum levels of specific micronutrients. There also is limited in vitro evidence demonstrating that micronutrients can influence the first step in carcinogenesis, binding of chemical carcinogens to DNA. These in vitro studies allow the determination of specific effects of individual micronutrients. The influence of micronutrients on DNA binding of aflatoxin B\(_1\) (AFB\(_1\)), a potent hepatocarcinogen, in mammalian cells is unknown. Woodchuck hepatocytes were used as a model to investigate the effects of vitamin A (all-trans retinol), C (ascorbic acid), ascorbyl palmitate (a synthetic lipophilic derivative of ascorbic acid), vitamin E (\( \alpha \)-tocopherol), and \( \beta \)-carotene on AFB\(_1\)-DNA binding.

Methods. Woodchuck hepatocytes were treated with 4 doses (0.080, 0.40, 2.0, and 10 \( \mu \)M) of \([3H]\)AFB\(_1\), or with different combinations of AFB\(_1\) and the vitamins for 6 hours, and adduct levels determined. Western blot analysis of protein extracts of treated cells was used to determine the effects of vitamin A and \( \beta \)-carotene on glutathione-S-transferase M1 levels.

Results. Vitamin A inhibited formation of AFB\(_1\)-DNA adducts in a dose-dependent manner throughout a concentration range of 34–122 \( \mu \)M by 40–80%. Vitamin C (0.080–10 \( \mu \)M) was much less effective than vitamin A as an inhibitor of AFB\(_1\)-DNA binding. Treatment with 6.0–48.3 \( \mu \)M ascorbyl palmitate reduced adduct levels at lower AFB\(_1\) concentrations but had no significant effect at higher AFB\(_1\) concentrations. \( \beta \)-Carotene and vitamin E enhanced covalent binding of AFB\(_1\) to DNA. Enhancement with \( \beta \)-carotene was observed when both tetrahydrofuran or liposomes were used as the administration vehicle. Western blot analysis indicated that neither the vitamin A nor \( \beta \)-carotene treatment affected glutathione-S-transferase M1 protein levels.

Conclusions. These results demonstrate that micronutrients play a complex role in the process of chemical carcinogenesis. Although protective effects were seen with several antioxidant vitamins, increased DNA adduct formation was observed with \( \beta \)-carotene and vitamin E. This antioxidant activity may be unrelated to the inhibition of DNA adduct formation. Additional studies are needed to understand the mechanism of enhanced adduct formation. Cancer 1994; 73:596–604.

Key words: chemical carcinogenesis, vitamins, aflatoxin B\(_1\), DNA adduct, glutathione-S-transferase.

Aflatoxins, especially aflatoxin B\(_1\) (AFB\(_1\)), the most abundant and potent naturally occurring form, are well-documented hepatic carcinogens in animals.\(^1,2\) A close correlation between aflatoxin exposure and incidence of hepatocellular carcinoma (HCC) also has been established by a number of ecologic correlation studies in areas of tropical Africa and Southeast Asia.\(^3,6\) Although hepatitis B virus (HBV) is regarded as the most important etiologic factor for HCC induction, a recent nested case-control study in Shanghai indicated a strong interaction between chronic HBV infection and aflatoxin exposure.\(^7\) For individuals who were chronic HBV carriers and had detectable urinary aflatoxin metabolites, the relative risk of HCC developing was 12.5 times greater than for those who had chronic HBV infection alone.

Hepatocarcinogenesis is initiated by covalent bind-
The formation of AFB₁-DNA adducts levels have been correlated with liver carcinogenicity in animal studies. The formation of AFB₁-DNA adducts can lead to mutations of proto-oncogenes and tumor suppressor genes, which play crucial roles in the pathogenesis of HCC and eventually result in neoplastic transformation. Although early detection of small HCC combined with surgical operation may prolong patient survival times, the prognosis of most patients with HCC is poor. Thus, elimination of aflatoxin exposure and HBV immunization are desirable approaches for prevention of aflatoxin-related HCC. However, identification of candidate chemopreventive agents to inhibit initiation by AFB₁ also has attracted increasing attention.

There is considerable evidence suggesting that a number of synthetic and natural compounds may modulate susceptibility to chemical carcinogens. Among the micronutrients, vitamins regulate a broad spectrum of physical and pathologic events. The protective roles of vitamin A and its analogs in carcinogenesis both in vitro and in vivo have been reviewed extensively. However, despite many epidemiologic studies suggesting that β-carotene may prevent the development of a wide range of cancers, few laboratory studies have investigated its role in carcinogenesis. Difficulties in the development of an appropriate animal model, with carotenoid absorption and tissue distribution as in humans, has hindered animal studies. Lack of an effective administration vehicle for β-carotene, a highly lipophilic compound, in cell culture systems also limits investigations on its in vitro effects.

Vitamin C inhibits chemical carcinogen-induced cell transformation and reduces the incidence of skin cancer in an animal model. A recent study also reported that a synthetic lipophilic derivative of vitamin C protected against the development of spontaneous HCC in mice. The protective role of vitamin E in cancer development is implied from its prominent antioxidant properties. However, few studies have evaluated the effect of vitamin E in experimental carcinogenesis. Epidemiologic studies on the association between serum levels of vitamin E and risk of cancer are inconsistent.

This study investigated the potential roles of vitamins A, C, and E and β-carotene on the initiation stage of AFB₁-induced hepatocarcinogenesis by investigating the covalent binding of AFB₁ to DNA in a woodchuck hepatocyte culture system. Both tetrahydrofuran and liposomes were used to administer β-carotene. Tetrahydrofuran has been reported to be an effective β-carotene vehicle in a cell culture system. Liposomes also have been established as a useful model membrane system for administering materials to the intracellular compartment. These two methods were used to assess the in vitro effects of β-carotene on the formation of AFB₁-DNA adducts.

Materials and Methods

Chemicals

AFB₁, dimethyl sulfoxide, bovine pancreatic RNase, all-trans-retinol (vitamin A), β-carotene, α-tocopherol (vitamin E), L-ascorbic acid (vitamin C), ascorbyl palmitate, and bovine heart phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). [³H]AFB₁ was purchased from Moravek Biochemicals (Brea, CA). Radioactive [³H]AFB₁ was diluted with the nonradioactive AFB₁ in dimethyl sulfoxide to obtain the required specific activity. Acetone, chloroform, phenol, isooamyl alcohol, and tetrahydrofuran without butyrate, and bovine heart phosphatidylcholine were products of Fisher Scientific Co. (Kent, WA). Proteinase K was obtained from Boehringer Mannheim Co. (Indianapolis, IN), cholesterol from Eastman Kodak Co. (Rochester, NY), and scintillation fluid (Liquiscint) from National Diagnostic Co. (Manville, NJ).

Cell Culture

Woodchuck hepatocytes, obtained from Dr. Charles Rogler, Albert Einstein Medical Center, New York, New York, were cultured in 100-mm tissue culture dishes in the presence of RPMI-1640 medium (Flow Laboratories, McLean, VA) containing 10% FCS (Sterile Systems, Logan, UT), supplemented with 50 units/ml of penicillin and 50 µg/ml of streptomycin.

Preparation of Stock Solutions of Vitamins and Liposomes

Stock solutions of vitamin A and ascorbyl palmitate were prepared in dimethyl sulfoxide, vitamin E in acetone, and vitamin C in RPMI medium containing 10% FCS. Solutions of β-carotene were prepared by being dissolved in tetrahydrofuran or incorporated into liposomes. All stock solutions were prepared immediately before use and an appropriate volume added to the culture medium so that the final concentration of solvent was less than 0.5%.

For the preparation of liposomes, we used a procedure that had been used previously for the liposome-mediated administration of retinoids to cells and membranes. Briefly, 10 mg of phosphatidylcholine and 5 mg of cholesterol were mixed with or without (for control purposes) an appropriate volume of β-carotene stock solution (2 mg/ml) in chloroform. The chloro-
form was evaporated under a stream of N₂, and 10 ml of RPMI medium was added. This mixture was sonicated at 50% maximal energy output using repeated sonication bursts of 30 seconds' duration until none of the lipid remained coating the glass tube and the solution took on a translucent appearance. The sonicated solution was kept on ice and flushed with N₂ throughout the entire sonication procedure. The β-carotene liposome preparation was diluted in an appropriate volume of culture medium immediately before use.

**AFB₁-DNA Binding Assay**

Before the effects of the vitamins were tested on the formation of AFB₁-DNA adducts, the possible cytotoxicity of each test compound and different combinations of AFB₁ and the test compounds for woodchuck hepatocytes were assessed by trypan blue exclusion. The highest concentration of each test compound was determined based on a cell viability of more than 90%. There was no significant difference in the cell survival between any treatment group and control cultures.

Approximately 8–10 days after the woodchuck hepatocytes were plated, confluent cultures were treated with four doses of AFB₁ (0.080, 0.40, 2.0, and 10 μM) containing 1 μCi [³H]AFB₁/ml (16–25 Ci/mmol) alone (controls) or various combinations of AFB₁ and the vitamins for 6 hours. After the treatment, cultures were washed twice with phosphate-buffered saline (PBS), and 1.5 ml of 10 mM Tris buffer (pH 7.9) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 0.4 M NaCl, and 0.2% sodium dodecyl sulfate was added. Hepatocytes were harvested by scraping and stored at −80°C until analysis. DNA was purified by standard phenol and chloroform/isoamyl alcohol (24:1) extractions and RNase treatment. DNA was dissolved in 0.5 ml PBS and the concentration determined by ultraviolet absorption at 260 nm. Radioactivity was determined by scintillation counting (LKB1215 Rackbeta, Pharmacia-LKB, Piscataway, NJ) and the modification level expressed as AFB₁ adducts/10⁶ nucleotides.

**Preparation of Hepatocyte Cytosols and Western Blot Analysis of Glutathione-S-transferase M1**

For determination of glutathione-S-transferase M1 (GST M1) protein levels, hepatocytes were treated with vitamin A or β-carotene and 10 μM of AFB₁ for 6 hours. Control cultures were treated with AFB₁ or solvent vehicles alone. After treatment, cells were washed twice, harvested by scraping and kept at −80°C. Frozen cell pellets were homogenized in ice-cold 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol with a polytron. Homogenates were centrifuged at 100,000 g for 1 hour at 4°C. The resulting supernatants were stored in aliquots at −80°C. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad, Melville, NY) with bovine serum albumin as the standard.

Cytosolic proteins (15 μg/lane) were electrophoresed on 12.5% acrylamide gels, transferred to nitrocellulose, and blocked overnight by incubation with 3% FCS. The antiserum against GST M1 (1:1000 dilution), provided by Dr. K. Cowan, National Cancer Institute, was incubated with the blots at room temperature for 1 hour. Blots were washed and stained with a Vectastain ABC anti-rabbit kit (Vector Laboratories, Burlingame, CA). The relative intensity of the GST M1 band was determined by densitometry.

**Results**

**Formation of AFB₁-DNA Adducts in Woodchuck Hepatocytes**

Woodchuck hepatocytes were treated in culture with [³H]AFB₁ for 6 hours. Throughout an AFB₁ concentration range of 0.080–10 μM, a linear relationship was observed between dose of AFB₁ and the amount bound to hepatocyte DNA (Fig. 1).
Figure 1. Dose-response relationship for AFB1-DNA adduct formation in woodchuck hepatocytes. Cells were treated for 6 hours. Binding data are expressed as adducts/10^6 nucleotides.

**Effects of Various Vitamins on AFB1-DNA Binding**

Covalent binding of AFB1-DNA was inhibited by vitamin A in a dose-dependent manner (Fig. 2). The presence of 34, 52, 87, and 122 μM vitamin A during treatment significantly reduced AFB1-DNA adduct levels by 40–80%. A maximum of 80% inhibition was observed at a dose of 122 μM vitamin A.

Figure 2. Effect of vitamin A on in vitro binding of AFB1 to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB1 in dimethyl sulfoxide (DMSO) (control) or with AFB1 and vitamin A in DMSO. Each treatment contained 0.2% DMSO. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB1 alone and are the average of two independent experiments.

Initial studies on the effect of β-carotene used tetrahydrofuran as the solvent (Fig. 3). The formation of AFB1-DNA adducts significantly increased in cells treated with β-carotene compared with control cells to a maximum of almost twofold. However, the degree of the enhancement was not related to β-carotene concentration. A similar enhancement of AFB1 binding was observed when liposomes were used to administer β-carotene (Fig. 4). The levels of AFB1-DNA adducts in cultures treated with β-carotene increased by 1.5 times compared with controls.

Figure 3. Effect of β-carotene using tetrahydrofuran as the administration vehicle on in vitro binding of AFB1 to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB1 in DMSO (control) or with AFB1 and β-carotene in tetrahydrofuran. Each treatment contained 0.05% DMSO and 0.45% tetrahydrofuran. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB1 alone and are the average of two independent experiments.

Vitamin C was nontoxic to woodchuck hepatocytes at concentrations as high as 10 mM. Throughout a concentration range of 0.080–10 mM, vitamin C suppressed the binding of AFB1 to hepatocyte DNA (Fig. 5). However, more than 50% inhibition of binding was found only at the highest vitamin C concentration, suggesting that it is much less effective than vitamin A in inhibiting AFB1-DNA binding. The inhibitory effect of vitamin C was dose-dependent at lower concentrations of AFB1 but not at higher concentrations. Because vitamin C is water soluble, it may not be efficiently absorbed into hepatocytes. This might account for the high concentrations required for significant inhibitory effects on adduct formation. Thus, the effect of ascorbyl palmitate, a synthetic lipophilic derivative of ascorbic acid, on binding of AFB1 to DNA was investigated (Fig. 6). Treatment with 6.0–48 μM ascorbyl palmitate re-
AFB₁ and vitamin A or β-carotene were evaluated by Western blot analyses. There were no significant differences in the intensities of the bands probed by an anti-GST M1 antiserum between vitamin A-treated and un-

**Figure 4.** Effect of β-carotene administered in liposomes on in vitro binding of AFB₁ to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB₁ (control) or with AFB₁ and β-carotene in liposomes. Each treatment contained 0.05% of DMSO and the same level of liposomes. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB₁ alone and are from a single experiment.

**Figure 5.** Effect of vitamin C on binding of AFB₁ to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB₁ DMSO (control) or with AFB₁ and vitamin C in RPMI medium. Each treatment contained 0.05% of DMSO. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB₁ alone and are the average of two independent experiments.

**Figure 6.** Effect of ascorbyl palmitate on binding of AFB₁ to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB₁ in DMSO (control) or with AFB₁ and ascorbyl palmitate in DMSO. Each treatment contained 0.3% of DMSO. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB₁ alone and are from a single experiment.

**Cellular Levels of β-Carotene and Vitamin A**

After treatment of hepatocytes for 6 hours, cellular β-carotene levels of 40 and 98 pmole/10⁶ cells were detected in extracts from hepatocytes treated with 7.5 and 14.9 μM of β-carotene, respectively. However, vitamin A was below the levels of detection in the same cell extracts. Although the stability of β-carotene in the hepatocytes was not investigated, these data indicated that β-carotene is absorbed, and it appeared that a linear relationship existed between media and cellular concentrations.

**Effects of Vitamin A and β-Carotene on Induction of GST M1**

Hepatic GST has been shown to play an important role in the inactivation of the cytochrome P450-generated AFB₁-epoxide. To investigate whether the effects of vitamin A or β-carotene on AFB₁-DNA binding could be attributed to modulation of GST M1 protein levels, extracts from cells treated with various combinations of
Figure 7. Effect of vitamin E on binding of AFB₁ to DNA. Woodchuck hepatocytes were treated for 6 hours with four doses of AFB₁ in DMSO (control) or with AFB₁ and vitamin E in acetone. Each treatment contained 0.05% of DMSO and 0.25% of acetone. Results are expressed as percent binding in vitamin-treated cells compared with control cells treated with AFB₁ alone and are the average of two independent experiments.

treated control cultures (Fig. 8). There also were no detectable differences in band intensities between β-carotene–treated and untreated control hepatocyte cultures (not shown).

Discussion
Liver cancer, largely HCC, is one of the most common cancers in the world and is associated with a remarkable geographic variation in incidence.³⁸ High-risk areas cluster in sub-Saharan Africa and Southeast Asia, where chronic HBV infection and dietary aflatoxin have been implicated as major risk factors.³⁸ Immunization against HBV and limiting exposure to AFB₁ are important for the control of HCC. However, hepatocarcinogenesis is a multistage and multifactorial process. In areas where HCC incidence is high, not only are HBV infection and aflatoxin exposure common, but malnutrition also is observed. There is a growing body of evidence from numerous in vitro and animal studies that many naturally occurring compounds in diet are important in chemical carcinogenesis.¹⁶,¹⁷ They may facilitate or impede the transport of carcinogens to their target sites, modify metabolic activation or inactivation of carcinogens, enhance or inhibit tumor cell proliferation, or alter host susceptibility.

Although there is no epidemiologic evidence to support a role for malnutrition in the development of HCC, it is reasonable to speculate that dietary deficiency may be important in aflatoxin-related HCC in high-risk areas. To address the issue of nutritional modulation in AFB₁ carcinogenesis by various vitamins, a woodchuck hepatocyte in vitro culture system was used in this study.

The crucial role of retinoids in cellular differentiation and proliferation has been well documented.¹⁵–¹⁶ The anti-initiating effect of retinoids on chemical carcinogen–induced sister chromatid exchange, DNA single strand breaks, cell transformation, and mutagenicity also have been demonstrated in a variety of in vitro studies.¹⁹–²¹,²³,²⁵,²⁹ A vitamin A-deficient diet enhanced liver and colon cancer and led to increased levels of AFB₁-DNA adducts in colon.³⁹ The current study provides additional evidence that vitamin A may be a potent inhibitor of the formation of AFB₁-DNA adducts, an essential step in AFB₁-induced hepatocarcinogenesis. However, these studies involved single dose treatments. The effect of vitamin A on the kinetics of DNA adduct formation and removal in animals chronically exposed to AFB₁ deserves additional study.

There are several possible explanations for the inhibitory effect of vitamin A on the in vitro binding of AFB₁ to DNA. Vitamin A may modulate the metabolism of AFB₁, scavenge cytochrome P450–generated reactive metabolites, interfere with the interaction between the metabolites and DNA, or alter the DNA repair process. Previous studies have demonstrated that vitamin A inhibited a variety of cytochrome P450 monooxygenase activities.²⁴,²⁵ It also has been reported that

Figure 8. Relative intensity of immunostaining of GST M1 in cytosolic proteins extracted from woodchuck hepatocytes treated with various combinations of AFB₁ and vitamin A. Cytosolic proteins (15 μg/lane) were electrophoresed and blotted onto nitrocellulose as described in Materials and Methods. Cytosolic proteins extracted from cells treated with: 0.15% DMSO, Lane 1; 10 μM AFB₁, lane 2; 87 μM vitamin A, lane 3; 122 μM vitamin A, lane 4; 10 μM AFB₁, and 87 μM vitamin A, lane 5; and 10 μM AFB₁ and 122 μM vitamin A, lane 6.
vitamin A deficiency caused a significant reduction in GST activity, which correlated with enhanced tobacco carcinogenesis in rats. In this investigation, despite the significant suppression in DNA modification by AFB_1 observed 6 hours after simultaneous treatment with AFB_1 and vitamin A, no significant change in the protein level of GST M1 was detected. Whether the effect of vitamin A is through some other mechanism remains to be elucidated. In vivo dietary β-carotene can be converted to retinol. Substantial evidence from experimental systems and epidemiologic studies suggests that β-carotene may have potential cancer chemopreventive activity. Previous studies have not satisfactorily demonstrated whether this activity was attributable to β-carotene or dependent on its conversion to retinoids.

Recent investigations on various carotenoids suggested that β-carotene may have an intrinsic biologic effect independent of its provitamin A activity on the enhancement of intercellular gap junctional communication and the expression of a major gap junction gene in the C3H/10T1/2 cell system. In contrast to the inhibitory effect of vitamin A on AFB_1-DNA binding in woodchuck hepatocytes, a significant enhancement of DNA binding during treatment with β-carotene was observed in this study. The enhancement effect of β-carotene was similar whether tetrahydrofuran or liposomes were used as the administration vehicle. Detectable levels of cellular β-carotene but not vitamin A were found in cultures treated with β-carotene for 6 hours. This strongly suggests that β-carotene exerts an effect distinct from vitamin A on AFB_1-DNA binding. Although there may have been low-level conversion of β-carotene into vitamin A, the effect should have been limited. There was no dose-dependent effect of β-carotene on the enhancement of AFB_1-DNA binding. This was not attributable to a nondose-dependent cellular uptake of β-carotene because a linear relationship between media β-carotene concentration and cellular levels was found. There is no established mechanism to explain the enhancement effect of β-carotene observed in this study. No effect of β-carotene on GST M1 protein level could be detected by Western blot analysis of cytosolic proteins. Additional study of the mechanism for the enhancement by β-carotene may provide important insights into its physiologic function.

Vitamin C has numerous and variable effects on cancer induction and growth. In this study, treatment with 0.080–10 mM vitamin C reduced the formation of AFB_1-DNA adducts. However, inhibition of binding by vitamin C was much less effective than that induced by vitamin A. This study also demonstrated that treatment with 24–48 μM ascorbyl palmitate inhibited AFB_1-DNA binding by approximate 50% at 0.080 μM AFB_1, whereas a relatively large dose of vitamin C was required to achieve the same effect. Ascorbyl palmitate is a synthetic lipophilic derivative of vitamin C and retains the antioxidant activity associated with vitamin C. It has been reported that ascorbyl palmitate suppressed chemical-induced skin tumor promotion in mice, and this inhibitory effect was more potent than that observed for vitamin C. The current data indicate that the introduction of a lipophilic capacity to vitamin C may increase its suppression of AFB_1-DNA adduct formation.

β-Carotene and vitamins C and E are among the most efficient antioxidants and scavengers of free radicals. There is considerable evidence that oxygen radicals are toxic by-products of many normal metabolic processes and may be involved in carcinogenesis. However, despite a broad range of carcinogens reported to be inhibited by antioxidants in a variety of experimental conditions, the experimental and epidemiologic evidence for the role of antioxidants in the prevention of cancer is inconsistent. In this study, vitamin C showed inhibitory activity on the binding of AFB_1 to DNA, whereas β-carotene and vitamin E enhanced the formation of the DNA adducts. These results suggest that the antioxidant activity may be unrelated to the inhibition of the covalent modification of DNA by AFB_1.

Increasing attention in cancer control has been paid to tertiary prevention defined as intervention to arrest or reverse a premalignant lesion. This study, using AFB_1-DNA adducts as an intermediate endpoint, provides information fundamental to the use of various vitamins as chemoprotective agents to reduce liver cancer incidence in high-risk areas. Although hepatocytes are the target of AFB_1 and have metabolic capability for its activation and detoxification, the complexity of the in vivo situation cannot be duplicated by the in vitro hepatocyte culture system. Additional in vivo studies are needed to investigate vitamin-modulated AFB_1-DNA binding. Methods for measurement of urinary aflatoxin metabolites and AFB_1-albumin adducts have been developed as biomarkers to quantify AFB_1 exposure in humans. These dosimetry methods will help to identify individuals at high risk for HCC. Prospective studies on the associations between serum vitamin levels and subsequent risk of HCC for individuals with exposure of aflatoxin may highlight the roles of vitamins in aflatoxin-induced human HCC.

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Vitamins and AFB,-DNA Binding/Yu et al.


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