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Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention

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To evaluate the efficacy of green tea polyphenols (GTPs) in modulating aflatoxin B_1 (AFB_1) biomarkers, a total of 352 serum samples and 352 urine samples collected from a 3 month chemoprevention trial with 500 mg GTPs, 1000 mg GTPs and a placebo were measured for AFB_1–albumin adducts (AFB–AA), aflatoxin M_1 (AFM_1) and aflatoxin B_1–mercapturic acid (AFB–NAC). Levels of AFB–AA at baseline were comparable for all three dose groups ($P = 0.506$). No significant differences were observed in AFB–AA levels in the placebo group over the 3 month period ($P = 0.252$). However, a significant reduction in AFB–AA levels was observed in the 500 mg group ($P = 0.002$). A marginally significant reduction in AFB–AA levels was also found in the 1000 mg group over the 3 month intervention period ($P = 0.051$). An analysis using a mixed-effects model indicated that the reduction in AFB–AA levels over time was dose and time dependent (dose–time interaction $P = 0.049$). There were no significant differences in median AFM_1 levels among the three study groups at the baseline ($P = 0.832$), 1 month ($P = 0.188$) and 3 months ($P = 0.132$) of the GTP intervention; however, reduction of 42 and 43% in median AFM_1 levels, as compared with the placebo, were found in 500 mg ($P = 0.096$) and 1000 mg ($P = 0.072$) groups at 3 months of the intervention. Significant elevations in median AFB–NAC levels and the ratio of AFB–NAC:AFM_1 were found in both 500 and 1000 mg groups compared with the placebo group at both 1 month ($P < 0.001$) and 3 months ($P < 0.001$) of GTPs intervention. These results demonstrate that GTPs effectively modulate AFB_1 metabolism and metabolic activation.

Introduction

Primary liver cancer, mainly hepatocellular carcinoma (HCC), is one of the most common cancers in southeast Asia and west Africa (1,2). The poor prognosis of this malignancy results in it being the third most common cause of cancer deaths in the world (1). In China, HCC is the second leading cause of cancer mortality with at least 350 000 deaths per year (3). There are several endemic regions in China, where HCC is the number one cause of cancer death and the annual incidence rate is usually higher than $50/10^5$ people (3). Southern Guangxi is one of these areas with the highest HCC incidence and mortality in China. In the period of 1997–2003, the mean morbidity rate of HCC in this area was $52.79/10^5$ (4). The median age of onset of this malignancy is between 35 and 45 years. Epidemiological studies have found that chronic infection with hepatitis B virus (HBV) and dietary aflatoxin (AF) exposure are major etiologic risk factors for HCC in this high-risk area (5).

AFs, produced predominantly by *Aspergilus flavus* and *Aspergillus parasiticus*, represent a group of fungal metabolites (mycotoxins) that have long been recognized as hazardous contaminants of food (6). Aflatoxin B_1 (AFB_1) is hepatotoxic and genotoxic and has been categorized as a known human carcinogen (Group I) (7–9). Chronic exposure to low levels of AFs has been proven to be one of the major risk factors in the etiology of HCC in several regions of Africa and Southeast Asia (10). More importantly, several nested case–control studies have demonstrated a synergistic interaction between AF and HBV for the risk of HCC (11,12). Therefore, the development and application of practical and highly effective intervention strategies for minimizing AF exposure and blocking carcinogenic effect is critical for reducing HCC risk, especially in high-risk populations.

Primary prevention strategies against major etiologic risk factors, such as vaccination of HBV in infants and food safety procedures to control AF contamination, have offered the best hope for lowering HCC rates in the world (13); however, they may require many years to implement. Therefore, a secondary prevention strategy, such as chemoprevention, has been widely considered a useful tool in high-risk populations (14). In fact, human chemoprevention trials aimed at lowering AF biomarkers have been conducted in high-risk populations of China (15–17) examining the use of oltipraz, chlorophyllin and green tea extracts.

Green tea or its major components, green tea polyphenols (GTPs), have been shown to be highly effective in inhibiting a variety of carcinogen-induced tumorigenesis in animal models for different target organ sites, including AF-induced liver tumors (18–20). Several studies observed that GTPs modulated AFB_1 metabolism, inhibited AFB_1 DNA binding and AFB_1-induced glutathione *S*-transferase (GST)-positive hepatocytes (18) and suppressed AFB_1-induced chromosome aberration in rats (21). GTPs are characterized by di- or trihydroxyl group substitution on the B-ring and the meta-5,7-dihydroxy substitution at the A-ring (22), which possesses strong antioxidant activity due to their metal chelating and free radical quenching ability.

Given the safety and efficacy of GTPs in multiple animal models, as well as its low cost, GTPs may be beneficial in modulating carcinogen metabolism and reducing oxidative stresses caused by carcinogen exposure, and therefore, reducing risk of HCC. To directly examine the possible adverse effect of GTPs in human subjects and study the modulation of GTPs on AF biomarkers in individuals at high risk of HCC, a randomized, double-blinded and placebo-controlled phase IIa chemoprevention trial was conducted in residents of Southern Guangxi, China (15). Our recent study showed that administration of GTP capsules to human subjects for 3 months effectively reduced levels of 8-hydroxydeoxyguanosine, the oxidative DNA damage biomarker (23). In this report, the efficacy of GTPs intervention was further evaluated by analyzing AF biomarkers in serum and urine samples collected prior to the study (baseline) and at 1 and 3 months of the study.

Materials and methods

Materials

GTPs were obtained from the USA–China joint venture Shili Natural Product Company (Guilin, Guangxi, China) and encapsulated by the Guangxi Pharmaceutical Company (Nanning, Guangxi, China). The purity of GTPs is higher than 98.5% according to the analysis by the Guangxi Standard Bureau. Each capsule of GTP contains 116 mg (-)-epigallocatechin-3-gallate, 53 mg epicatechin-3-gallate, 25 mg (-)-epicatechin 25, 19 mg (-)-epigallocatechin, 24 mg gallocatechin gallate and 11 mg catechin according to the analysis using high-performance liquid chromatography (HPLC)–electro-CoulArray detection and HPLC–ultraviolet methods. [^{3}H]-AFB_1 (28 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Monoclonal antibodies 2B11 and 2F5 were

Abbreviations: AF, aflatoxin; AFB_1, aflatoxin B_1; AFB–AA, aflatoxin B_1–albumin adducts; AFM_1, aflatoxin M_1; AFB–NAC, aflatoxin B_1–mercapturic acid; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; GTP, green tea polyphenol; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; IAC, immunoaffinity column; MeOH, methanol; PBS, phosphate buffering solution; RIA, radioimmunoassay; SD, standard deviation.

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kindly provided by Dr G.N.Wogan at Massachusetts Institute of Technology. Aflatoxin B_1–mercapturic acid (AFB–NAC) was synthesized as reported previously by Scholl *et al.* (24). AFB_1, AFB_2, aflatoxin M_1 (AFM_1), albumin and creatinine detection kits were purchased from Sigma–Aldrich Chemical Company (St Louis, MO). Oasis® HLB cartridges were products of Waters Corporation (Milford, MA). All organic solvents used were of HPLC grade. Other chemicals and reagents were purchased commercially at the highest degree of purity available.

Study sites, subjects and protocols

For human subject protection, this study was approved by the Institutional Review Boards at Texas Tech University and Guangxi Cancer Institute. The overall study design was described previously (15). Briefly, the study site includes two villages (Sanhe and Zhuqing), located 45 km southwest of Fusui county, Guangxi Zhuang Autonomous Region, China. The site is a rural farming community with ~7500 residents and it belongs to the Qujiu Township, one of the three townships with the highest incidence and mortality of liver cancer (~100/100 000) in Fusui county. Twelve hundred blood samples were screened for AFB_1–albumin adducts (AFB–AA) and HBV infection status, and 120 voluntary residents were enrolled into this trial. The recruiting criteria included healthy adults with positive serum hepatitis B virus surface antigen and AFB–AA, aged 20–55, normal liver function test (alanine aminotransferase < 80 U/ml), alpha-fetoprotein negative, no personal history of cancer, no use of prescribed medications and no pregnancy and lactation for female participants. Informed consent was obtained from each participant before they were randomly assigned to one of three study groups. Baseline blood and urine samples were collected before the trial began. Randomization was successful: no significant differences with regard to age, gender and baseline AFB–AA levels were found among groups (15). Participants were instructed to take four capsules daily containing either 500 mg GTPs (low dose, $n = 40$), 1000 mg GTPs (high dose, $n = 40$) or 1000 mg medicinal starch as the placebo control ($n = 40$). The doses of 500 and 1000 mg GTPs were chosen to be equivalent to two and four 500 ml cups of green tea drink, respectively. Follow-up visits were made every other day at the participant's house to record possible adverse effect complaints and to count the remaining capsules for adherence assessment. No severe adverse effects were recorded according to the clinical tests of blood and urine samples at each collection, including blood counts, blood chemistry, alanine aminotransferase, aspartate aminotransferase, urinary protein, glucose, blood and others (15). An excellent person-time compliance (99.5%) was achieved (15).

Sample collection

In addition to regular epidemiological questionnaires, blood samples (5 ml for serum and 5 ml for plasma) and 24 h urine samples were collected at 1 and 3 months of the trial. Serum, plasma and blood cells were immediately separated and stored at −20°C in the village clinics. Twenty-four hour urine samples were collected in the morning, noon and evening in 1 day and kept in amber bottles containing ascorbic acid (20 mg/ml) and ethylenediaminetetraacetic acid (0.1 M). Aliquots of urine samples (50 ml) were treated with 500 mg ascorbic acid and 12.5 mg ethylenediaminetetraacetic acid for biomarker analysis. All samples were shipped frozen to Texas Tech University and the laboratory personnel who performed analysis were blinded to sample sources. Sample collection, storage and shipment complied with guidelines of both Chinese and USA governments.

Determination of serum levels

A quantitative radioimmunoassay (RIA) procedure with monoclonal antibody 2B11 was used to measure AFB–AA (25). Briefly, serum albumin was concentrated, digested and dissolved in 100 µl of phosphate buffering solution (PBS) and added to monoclonal antibody 2B11, which was dissolved in 100 µl of PBS with 10% horse serum. A tracer solution (100 µl) containing 12 000 d.p.m. of [^{3}H] AFB_1 was then added. After incubation for 2 h, 300 µl of saturated ammonium sulfate was added and the sample was mixed and incubated for 15 min. This solution was centrifuged for 15 min at 9800*g* and the radioactivity remaining in the supernatant was determined by liquid scintillation spectrometry. Non-specific inhibition in the assay was determined by processing pooled normal rat serum (Sigma–Aldrich Chemical Company). The average value was subtracted from those of the study samples in calculating AFB–AA levels. Standard curves were determined using a non-linear regression method as described previously (26). Values were expressed as the amount of AFB_1/mg albumin, measured colorimetrically with bromocresol green at 628 nm (Sigma–Aldrich Chemical Company). As the purpose of quality control, AFB_1-treated rat serum with known AFB–AA concentration was spiked to normal human serum and was concurrently processed for RIA. The limit of detection is 0.01 pmol AFB_1/mg albumin.

Determination of AFB_1 metabolites in urine

A modified immunoaffinity–HPLC–fluorescence detection method was used to measure the AFB_1 metabolites in urine (12,27). Briefly, 10 ml of urine sample (in some samples 1 ng AFB_2 was spiked as internal standard) was pH adjusted with ammonium formate (1 M, pH 4.5) and loaded into a conditioned Oasis® HLB column. The column was washed with 10 ml of water and 10 ml of 5% methanol (MeOH) before being eluted by 3 ml of MeOH. The eluate was dried under ultra high purity N^2 and reconstituted with 2 ml of PBS (pH 7.2). The reconstituted urine extract was then loaded into the prepared and conditioned immunoaffinity column (IAC) including both 2B11 and 2F5 monoclonal antibodies at a flow rate of 0.3 ml/min. The affinity column was then washed twice with 5 ml of PBS (pH 7.2) and once with 10 ml of water to remove non-specifically bound materials. AF derivatives were eluted from the IAC with 2 ml of 60% dimethylsulfoxide in water. The elution was diluted with 8 ml of water and loaded into a conditioned Oasis® HLB column and washed with 10 ml of water and 10 ml of 5% MeOH. The concentrated AF derivatives were eluted with 3 ml of MeOH and were reduced to ~100 µl under ultra high purity N^2 and mixed with 5 mM triethylammonium formate (pH 3.0) to reach 400 µl before analysis by HPLC.

Urinary AFM_1 and AFB–NAC were analyzed by reversed-phase HPLC on an Agilent 1100 system consisting of a diode-array ultraviolet detector (wavelength 362 nm) connected in series with a fluorescence detector (366 nm excitation and 436 nm emission). The HPLC column used was a C18 5 µm (150 × 4.6 mm) Microsorb analytical column (Varian, Palo Alto, CA). Chromatographic separation was obtained by a 5–25% ethanol linear gradient in water generated over a 25 min period followed by isocratic elution with 25% ethanol in water, all at a flow rate of 1 ml/min. The mobile phase was buffered with 5 mM triethylammonium formate (pH 3.0) and the column temperature was maintained at 35°C. The eluted peaks were integrated and AFB_1 metabolites were quantitated with the standard curves for each metabolite or biomarker. Authentic AFB_1 metabolites were eluted at 15.5 min for AFB–NAC and 18.9 min for AFM_1. The limit of detection for the method was 1.0 pg for AFM_1 and 5 pg for AFB–NAC. Urinary creatinine concentration was determined with the Diagnostic Creatinine Kit from Sigma–Aldrich Chemical Company according to the manufacturer's instruction. Recovery was 90% for spiked AFM_1 (0.25–5 ng), 83% for spiked AFB–NAC (0.5–10 ng) and 55–65% for spiked AFB_2 (0.5–5 ng) for this method.

Statistical analysis

All data generated were stored in an Excel database and analyzed with SAS software version 9.3 (SAS Institute, Cary, NC). Median, mean, standard deviations (SDs) and range were calculated for concentrations of AFB–AA, AFM_1 and AFB–NAC and the values were expressed as median and mean ± SD unless otherwise stated. To assess the efficacy of GTPs intervention, the statistical evaluation focused on the comparisons of different treatments and different time points. To evaluate the overall effects of dose, time and the dose × time interaction on AFB–AA, AFM_1, AFB–NAC and AFB–NAC/AFM_1, a non-parametric mixed-effects model was used (28). To facilitate interpretation of the mixed-effects model results, repeated measures analysis of variance were performed to compare time periods within a dose group. Cross-sectional analyses of variance were performed to compare groups within a time period. For parameters that were normally distributed, analysis of variance and Bonferroni corrected *t*-tests were used. For parameters that were not normally distributed, a Kruskal–Wallis test or Wilcoxon rank sum test was used. A P value of <0.05 (two tailed) was considered significant.

Results

Sample collection over the study period

A total of 120 human subjects were recruited and 116 human subjects (96.7%) completed the 3 month intervention trial. Among the three time points of sample collection, 352 serum samples and 352 urine samples were collected from the participants.

Modulation of serum AFB–AA levels

All 352 serum samples collected over the 3 month study period were analyzed and all samples (100%) had detectable AFB–AA. Average levels (median and mean ± SD) and the range of serum AFB–AA in the three treatment groups at different time points are shown in Table I. There were no differences in AFB–AA level among the treatment groups at baseline ($P = 0.506$). The distributions of AFB–AA throughout the study duration are shown in Figure 1. No statistically significant differences were observed in AFB–AA levels in the placebo group over the 3 month period ($P = 0.252$). However,

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Table I. Levels of AFB_1 biomarkers in GTPs intervention study[a]

Time (month)	Placebo	GTP 500 mg	GTP 1000 mg
AFB–AA (pmol/mg albumin)			
0	0.92 0.91 ± 0.24 (0.48–1.41)	0.89 0.92 ± 0.28 (0.36–1.63)	0.91 0.92 ± 0.24 (0.49–1.55)
1	0.90 0.96 ± 0.32 (0.32–1.70)	0.94 0.96 ± 0.23 (0.65–1.54)	0.77[b] 0.82 ± 0.27 (0.35–1.40)
3	0.85 0.85 ± 0.26 (0.16–1.40)	0.74[c] 0.79 ± 0.19 (0.26–1.19)	0.78 0.80 ± 0.34 (0.50–1.40)
AFM_1 (pg/mg creatinine)			
0	7.69 59.41 ± 141.99 (0.42–730.2)	6.02 60.85 ± 148.17 (0.59–746.10)	6.75 40.12 ± 77.42 (0.52–308.27)
1	13.87 61.67 ± 145.74 (0.52–881.39)	8.29 15.03 ± 15.82 (0.38–64.27)	14.27 20.06 ± 16.14 (0.77–51.50)
3	11.24 78.66 ± 243.32 (0.24–1276.25)	6.51 16.12 ± 45.07 (0.18–222.35)	6.41 25.95 ± 73.03 (0.12–338.85)
AFB–NAC (pg/mg creatinine)			
0	5.93 8.67 ± 9.83 (0.43–41.15)	6.34 10.31 ± 12.38 (0.38–50.77)	6.54 9.32 ± 11.32 (0.60–67.71)
1	5.50 9.95 ± 12.97 (0.09–57.92)	37.95[c] 79.53 ± 89.48 (1.57–362.47)	43.09[c] 79.48 ± 93.07 (0.30–465.62)
3	4.27 6.11 ± 8.72 (0.43–50.58)	72.29[c] 97.76 ± 100.03 (11.32–501.48)	61.34[c] 96.60 ± 117.45 (18.20–560.30)
AFB–NAC/AFM_1			
0	1.01 2.53 ± 3.93 (0.01–15.05)	1.35 3.13 ± 4.70 (0.01–17.96)	1.05 2.54 ± 3.37 (0.02–13.40)
1	0.43 2.19 ± 4.02 (0.01–15.36)	4.40[c] 22.05 ± 58.35 (0.08–333.80)	2.86[d] 8.21 ± 15.94 (0.40–89.29)
3	0.26 5.39 ± 6.88 (0.01–30.71)	6.72[c] 16.45 ± 22.94 (0.03–109.33)	6.92[c] 12.46 ± 17.17 (0.01–95.22)

[a]Data are presented in the form: median, mean ± SD and (range).
[b]$P = 0.05$ as compared with the baseline.
[c]$P < 0.01$ as compared with the baseline and the placebo.
[d]$P < 0.05$ as compared with the baseline.

statistically significant difference in AFB–AA levels was observed in the 500 mg GTPs group over the 3 month period ($P = 0.002$). A marginally significant difference in AFB–AA levels was also found in the 1000 mg GTPs group over the 3 month intervention ($P = 0.051$). As shown in Table I, compared with levels at baseline and the placebo, a significant decrease in AFB–AA level ($P < 0.01$) was found in the 500 mg GTPs group at the 3 months of intervention. As compared with levels at baseline, marginally significant decreases in the adduct levels were also found in the 1000 mg GTPs group at both 1 month ($P = 0.050$) and 3 months ($P = 0.079$) of intervention. The non-parametric mixed-effects model showed significant effects of time ($P = 0.003$) and dose–time interaction ($P = 0.049$) for reducing serum AFB–AA levels (Table II) and this reduction was attributed to GTP intervention. In addition, no significant gender difference was found (data not shown).

Modulation of urinary AFM_1 level

A total of 352 urine samples collected over the 3 months GTP intervention were analyzed for AFM_1. About 95% (334/352) of the

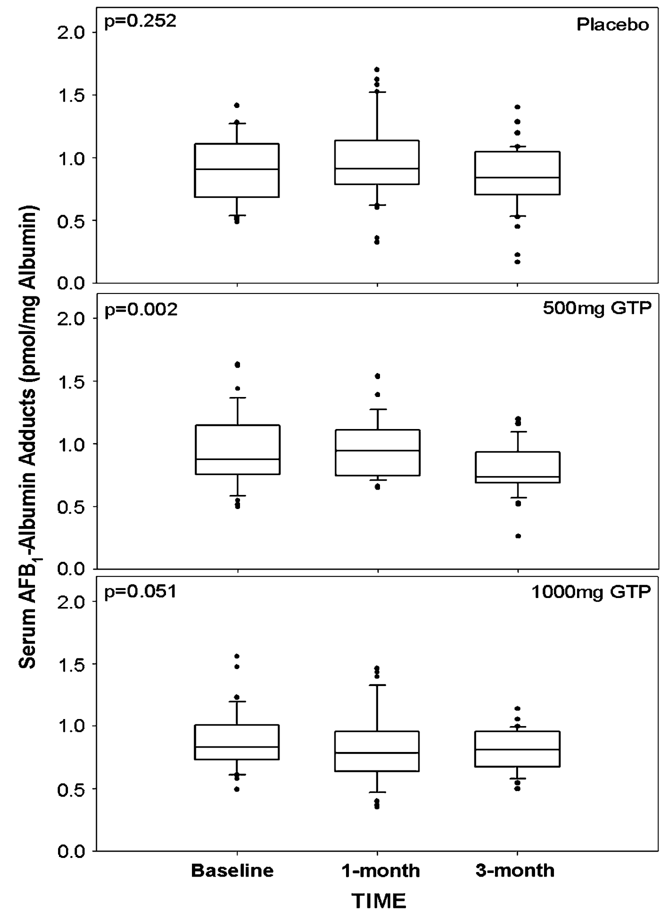

Fig. 1. Serum AFB–AA levels over 3 months GTP intervention. The box plots shows distribution of serum AFB–AA levels in each group at each time point. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

samples had detectable AFM_1 and no significant differences in detection rate (frequency) among the three treatment groups were found. As shown in Table I, no statistically significant difference was found in average levels (median and mean ± SD) and the range of AFM_1 in the three study groups at the baseline, 1 and 3 months of the intervention. The distribution of urinary AFM_1 levels in these three groups is shown in Figure 2. Because the AFM_1 data are highly skewed, non-parametric analysis was applied for all statistical evaluations. There were no significant differences in median AFM_1 levels among the three study groups at the baseline ($P = 0.832$), 1 month ($P = 0.188$) or 3 months ($P = 0.132$). However, as compared with the placebo groups, 42 and 43% reductions in median AFM_1 levels were found in the 500 mg GTPs group ($P = 0.096$) and the 1000 mg GTPs group ($P = 0.072$) at 3 months of intervention, respectively. The non-parametric mixed-effects model showed a significant time effect on urinary AFM_1 levels (Table II), but no dose or dose–time interaction was found. In addition, no significant gender difference was found (data not shown).

Modulation of urinary AFB–NAC levels

A total of 352 urine samples collected over 3 months intervention study were simultaneously analyzed for AFB–NAC. Again, ~95% (336/352) of the samples had detectable AFB–NAC and no significant difference was found in the detection rate (frequency) among the three treatment groups. Average levels (median and mean ± SD) and the range of AFB–NAC in three study groups at baseline, 1 and 3 months are also presented in Table I. The distribution of urinary AFB–NAC levels in these three groups is shown in Figure 2. No statistically

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Table II. Non-parametric mixed-effects model analysis

Effect	Serum AFB–AA	Urinary AFM_1	Urinary AFB–NAC	Urinary AFB–NAC/AFM_1
Dose	0.681 ($P = 0.506$)[a]	0.757 ($P = 0.465$)	74.993 ($P < 0.001$)	21.797 ($P < 0.001$)
Time	6.371 ($P = 0.003$)	57.974 ($P < 0.001$)	72.164 ($P < 0.001$)	76.601 ($P < 0.001$)
Dose × time	2.550 ($P = 0.049$)	0.541 ($P = 0.597$)	25.382 ($P < 0.001$)	8.217 ($P < 0.001$)

[a]Statistic box-approximation value (*P* value).

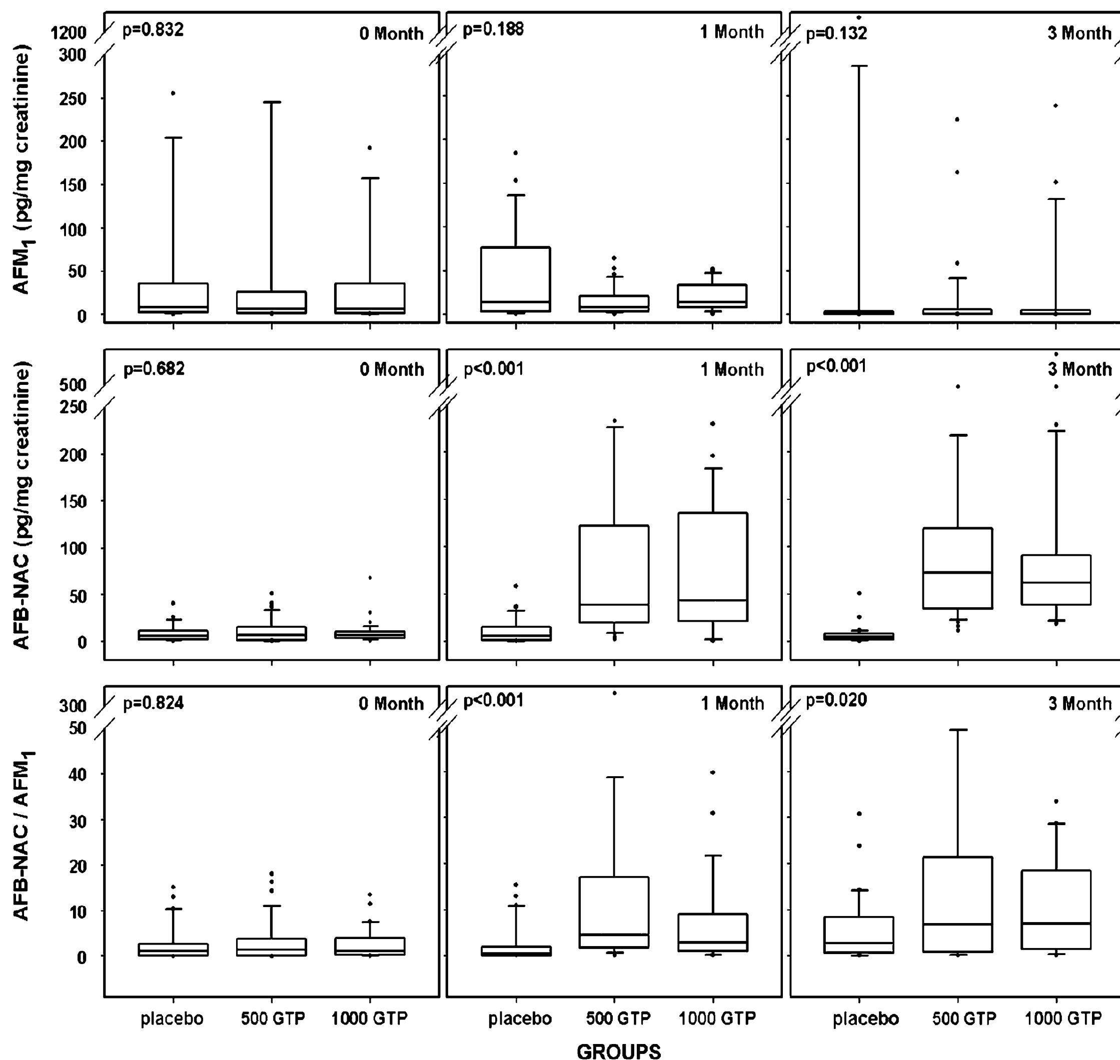

Fig. 2. Urinary AFM_1, AFB–NAC and AFB–NAC/AFM_1 levels over 3 months GTP intervention. The box plots show distributions of urinary AFM_1, AFB–NAC and AFB–NAC/AFM_1 levels in each group at each time points. The box values ranged from 25 to 75 percentile of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 percentile and from 75 to 95 percentile, respectively.

significant difference was found among the baseline ($P = 0.682$) or the placebo group over 3 months period; however, there were statistically significant elevations in median AFB–NAC levels among the three study groups at 1 month ($P < 0.001$) and 3 months ($P < 0.001$) of GTP intervention (Figure 2). Median AFB–NAC levels increased ~7- and 17-fold in the 500 mg GTPs group at 1 and 3 months, respectively. Approximately 8- and 14-fold increases of median AFB–NAC levels were observed in the 1000 mg GTPs group at 1 and 3 months, respectively. The non-parametric mixed-effects model showed significant effects for dose, time and the dose–time interaction on urinary AFB–NAC levels (Table II). In addition, no significant gender difference was found (data not shown).

Modulation of AFB metabolic pattern

Because AFM_1 and AFB–NAC are major phase 1 and phase 2 metabolites of AFB_1, the ratio of AFB–NAC:AFM_1 was postulated to better reflect the overall modulation of AFB_1 metabolism by incorporating both phase 2 and phase 1 metabolites. As shown in Table I, no statistically significant difference was found among the baseline ($P = 0.824$) or the placebo group over 3 months period. However, there were statistically significant elevations in the median ratio of AFB–NAC:AFM_1 among the three study groups at 1 month ($P < 0.001$) and 3 months ($P = 0.020$) as demonstrated in Figure 2. Approximately 10- and 26-fold increases of the median ratio of AFB–NAC:AFM_1 were

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observed in the 500 mg GTPs group at 1 and 3 months (Table I), respectively. Approximately 7- and 27-fold increases of the median ratio of AFB–NAC:AFM_1 were found in the 1000 mg GTPs group at 1 and 3 months, respectively. The non-parametric mixed-effects model also showed significant effects on dose, time and the dose–time interaction on the ratios (Table II). It is apparent that treatment with GTPs enhanced the AFB_1 phase 2 detoxification pathway. Representative chromatograms of HPLC–fluorescence detection for AFB–NAC and AFM_1 are shown in Figure 3.

Discussion

It is well known that the toxic and carcinogenic effects of AFB_1 are manifested after phase 1 metabolism by endogenous cytochrome P450 enzymes, such as CYP 1A2, 2A6 and 3A4 (7). These enzymes catalyze the formation of various oxidative derivatives, including AFM_1 and an unstable and highly reactive ultimate carcinogen, AFB_1-8,9-epoxide that can covalently bind to macromolecules and form adducts (7). Two major macromolecular adducts are the AFB_1–N^7–guanine adduct in DNA (29) and the lysine adduct in serum albumin (30). AFB_1-8,9-epoxide is further metabolized by phase 2 enzymes, GSTs, to produce AFB–NAC, a detoxification metabolite excreted in urine (24,27). Application of well-defined AFB_1 metabolic pathways and specific AFB_1 biomarkers to evaluate efficacy of chemopreventive agents such as GTPs could provide mechanistic information for human intervention trials as described in previous studies with oltipraz (27,31,32), which was initially identified as candidate chemopreventive agent based on its phase 2 enzyme-inducing property.

AFB_1-specific biomarkers currently used in human and animal studies include AFM_1, AFB–NAC and AFB–N^7–guanine in urine and AFB–AA in serum (31,33). The AFB–AA, compared with urinary AFB_1 metabolites, serves as a very important biomarker because its longer *in vivo* half-life may reflect integrated exposures over longer time periods (34). From a practical perspective relevant to epidemiological studies, the measurement of serum AFB–AA offers a rapid and facile approach that can be used to screen very large numbers of people, especially for population studies (35). The AFB–AA is also the most reliable molecular biomarker for studying human exposures to AFB_1. Highly significant associations between AFB–AA levels and AFB_1 intake were found in human populations from several regions of the world (36–38). Using various analytical techniques, AFB–AA was detectable in almost 100% of sera from adults and in 12–100% of sera from children in China and various African countries (36–38). In addition to studying AFB_1 exposure, AFB–AA has been used as a biological response indicator of acute and chronic aflatoxicosis in Africa (38), risk of HCC in Taiwan (39), China (5,11,12) and Africa (38) and infectious disease-linked immune suppression (40,41). Moreover, AFB–AA has been regularly used as the surrogate efficacy biomarker of AFB_1 exposure for assessment of different therapeutic/intervention agents and techniques in human intervention trials (17,42).

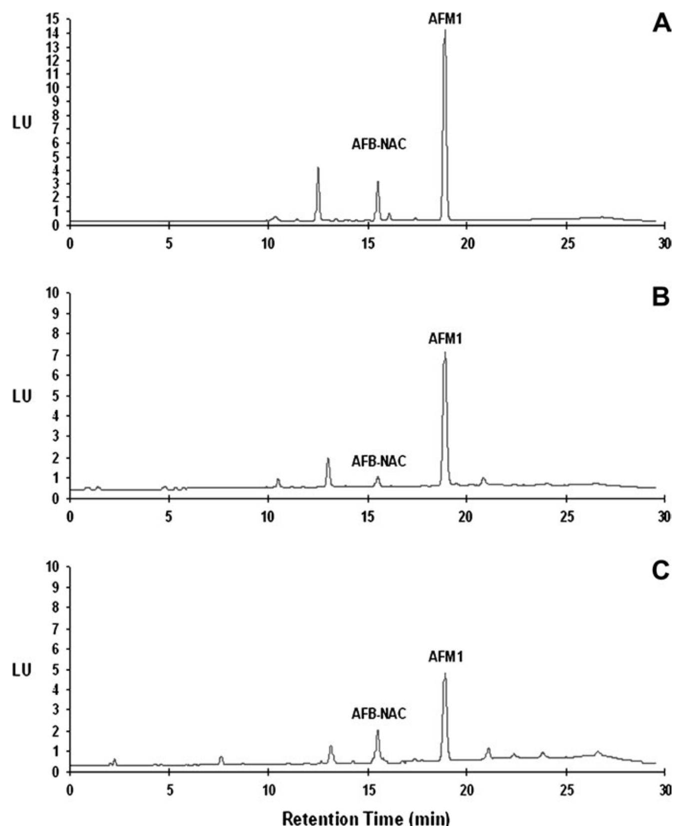

Fig. 3. Chromatograms of HPLC–fluorescence detection for urinary AFB–NAC and AFM_1. (**A**) Authentic AFB–NAC and AFM_1 standards spiked to a non-detectable human urine sample. (**B**) A human urine sample collected at 0 month (baseline) and (**C**) a human urine sample collected at 3 months after GTP intervention.

In this study, high levels of serum AFB–AA were observed in the participants at baseline before GTPs intervention. These levels were comparable with levels found in populations at high risk for liver cancer in China (25,34,36) and confirmed high dietary exposure to AF in the study population. Daily GTPs administration significantly reduced levels of serum AFB–AA (Table I). A significant reduction (>15%) was observed in the 500 mg GTPs group at 3 months compared with levels of the placebo and the baseline (Table I and Figure 1). Decreases in this biomarker level were also observed in the 1000 mg GTP groups at 1 and 3 months after the intervention.

There are four methods currently available for measurement of serum AFB–AA: RIA using monoclonal antibodies 2B11 or 2A4B3 (17,25,34); enzyme-linked immunosorbent assay (ELISA) using polyclonal or monoclonal antibodies (35,38,39); IAC–HPLC–fluorescence method (25,37,38) and the recently developed liquid chromatography/mass spectrometry/mass spectrometry method with or without IAC purification (43,44). Among these four methods, RIA method has been widely validated in animal studies (45,46), human cross-sectional studies (34,37,41), human longitudinal studies (25), HCC case–control studies (47,48) and human chemoprevention studies with oltipraz (17). Correlations between ELISA and HPLC–fluorescence (38), between ELISA and liquid chromatography/mass spectrometry/mass spectrometry (49), between RIA and HPLC–fluorescence (25) and between RIA and ELISA (50) have been evaluated. Overall, data generated by these methods were highly correlated, e.g. RIA versus ELISA ($r = 0.75$, $P < 0.01$), RIA versus HPLC ($r = 0.87$, $P < 0.01$) and 2B11 RIA versus 2A4B3 RIA ($r = 0.86$, $P < 0.01$) (50).

AFM_1 is a metabolite of AFB_1 that is prevalent in urine and milk and its formation from parent AFB_1 is catalyzed mainly by hepatic CYP 1A2 in humans (27). The excretion of AFM_1 in urine represents recent AFB_1 exposure (i.e. within 24–48 h). Thus, AFM_1 levels in urine are used as a short-term biomarker of AFB_1 exposure (37). Both serum AFB–AA and urinary AFM_1 have been extensively characterized and validated as biomarkers for AFB_1 exposure in many human populations, which correlated well with dietary intake of AFB_1 (33,37) and the risk of human HCC (33). Concurrent with reductions in serum AFB–AA levels after GTP intervention, urinary AFM_1 levels were reduced (up to 43% in median level) at 3 months of intervention, which is comparable with the reduction rate of 55% in the median level of AFB_1–N^7–guanine, another short-term biomarker of AFB_1 exposure, after 3 months intervention with 100 mg chlorophyllin (16).

As demonstrated by many previous studies, the chemopreventive action of a variety of natural products or drugs is associated with the induction of carcinogen detoxification enzymes (51). Induction of phase 2 enzymes plays a crucial role in providing a barrier against exogenous chemical carcinogenic effects (52). AFB–NAC is the major detoxifying metabolic product of AFB_1-8,9-epoxide (24,27). GTPs intervention significantly elevated levels of AFB–NAC in urine excretion in both the 500 and 1000 mg groups, which suggests that activity of GSTs was greatly induced. The increase in the

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AFB_1–NAC:AFM_1 ratio in GTPs-treated groups further demonstrated effective modulation of GTPs on induction of the phase 2 detoxifying pathway in AFB_1 metabolism. This finding is consistent with a recent finding that GTPs increased the activity of GSTs in 42 human subjects who underwent 4 weeks of intervention with polyphenon E (53).

Results of this study clearly show that GTP intervention effectively modulated AFB_1 metabolism as well as metabolic activation, as demonstrated by the decreased serum levels of AFB–AA and urinary levels of AFM_1. This suggests that GTPs may inhibit phase 1 metabolic enzymes, such as CYP 1A2, 2A6 and 3A4. Based on the moderate effect in reducing levels of AFM_1, a major CYP 1A2 metabolite, in urine, GTPs seems to be a moderate or reversible inhibitor of 1A2 enzyme. This is different from the modulation effect of oltipraz, which is a potent and perhaps irreversible inhibitor of 1A2 (27).

While data from this study clearly demonstrated that GTPs modulate AFB_1 metabolism and metabolic activation, large variations in levels of individual AFB_1 biomarkers were found, especially for urinary AFM_1 and AFB–NAC levels. The variability of these biomarkers in study participants may be attributed to seasonal changes in food contamination in the region, as well as to genotypic or phenotypic variations on specific metabolic enzymes and individual susceptibility. Another issue that has been raised is the analytical limitation of the current study, due to the use of antibody-based RIA and IAC–HPLC techniques, which compared with recently developed liquid chromatography/mass spectrometry/mass spectrometry method, the specificity and sensitivity may be lower. Levels of AF biomarkers measured by antibody-based methods are usually higher than those measured through the LC/MS method in which isotope internal standard was coupled. It would be potentially important in ultimate risk assessment for AF exposure if a global collaborative study is set up for addressing the issue on various results generated by different analytical methods, using LC/MS method as the standard. In any sense, LC/MS method should be incorporated into all future AF exposure and intervention studies, at least used as a confirmatory step.

Safety and efficacy are the two most important criteria for assessing potentially chemopreventive agents. The safety of GTPs has been well documented in animal and human studies (19,20), including this 3 months trial (15). Results from this study show the efficacy of GTPs through modulation of AFB_1 metabolism, metabolic activation and detoxification. As summarized in Figure 4, there are two major metabolic pathways for AFB_1: phase 1 metabolism and metabolic activation and phase 2 detoxification (27,33). AFM_1 and AFB_1-8,9-epoxide are the major phase 1 metabolic products and AFB_1–N^7–guanine in tissues and urine and AFB–AA in serum are specific biomarkers for AFB_1 metabolic activation. AFB–NAC is the major phase 2 detoxification product of AFB_1-8,9-epoxide. GTP intervention significantly blocked phase 1 metabolism and metabolic activation of AFB_1 and greatly induced phase 2 detoxifying enzymes, which led to increased formation of AFB–NAC excreted in urine. Results from this study as well as previous studies (23,53) will help to define mechanistic roles of GTPs in cancer chemoprevention.

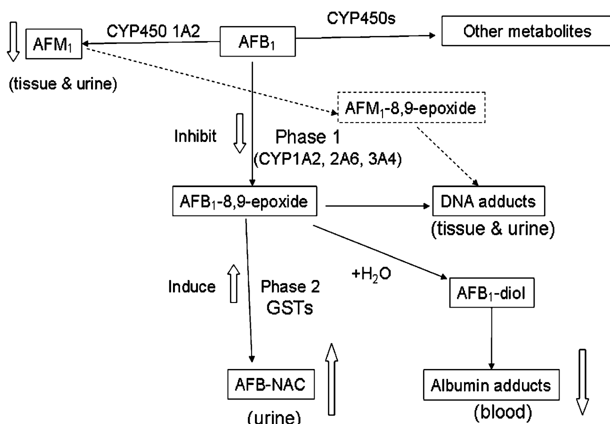

Fig. 4. AFB_1 metabolism, metabolic activation, detoxification, biomarkers and possible mechanisms of GTP modulation.

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