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Naturally occurring chlorophyll derivatives inhibit aflatoxin B_1-DNA adduct formation in hepatoma cells

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ABSTRACT

The inhibitory effects of four chlorophyll derivatives (chlorophyllide [Chlide] a and b and pheophorbide [Pho] a and b) on aflatoxin B_1 (AFB_1)-DNA adduct formation, and on the modulation of hepatic glutathione *S*-transferase (GST) were evaluated in murine hepatoma (Hepa-1) cells. Enzyme-linked immunosorbent assay showed that pretreatment with Chlide or Pho significantly reduced the formation of AFB_1-DNA adducts, and that Pho was the most potent inhibitor. However, wash-out prior to adding AFB_1 totally eliminated inhibition by Childe and partially eliminated inhibition by Pho, indicating that the inhibitory effect of Chlide, and to some extent Pho, was mediated through direct trapping of AFB_1. Furthermore, spectrophotometric analysis showed that Pho treatment could increase GST activity in Hepa-1 cells. These observations indicate that the chlorophyll derivatives studied may attenuate AFB_1-induced DNA damage in the Hepa-1 cell by direct trapping of AFB_1. Pho provided additional protection not only by direct trapping, but also by increasing GST activity against hepatic AFB_1 metabolites.

1. Introduction

Epidemiological studies have demonstrated that high consumption of fruits and vegetables is associated with reduction of cancer risk in humans [1]. The beneficial effects of fruits and vegetables have been partly attributed to the presence of numerous phytochemicals. However, many of these phytochemicals elicit chemopreventative effects in experimental animals only at doses far above the concentrations commonly encountered in the human diet. One promising exception may be chlorophyll and chlorophyll-related chemicals.

Chlorophylls are naturally catabolized into two related chemicals, chlorophyllide and pheophorbide (Fig. 1). The chlorophylls found in green vegetables are made of a porphyrin ring to which is attached a long-chain phytol tail, and in which a Mg atom is chelated. Removal of the phytol tail from chlorophyll forms chlorophyllide (Chlide), and the removal of both the phytol tail and the chelated Mg atom forms pheophorbide (Pho). The chlorophylls chlorophyll a and chlorophyll b vary slightly in the chemical structure of their porphyrin ring and are converted into chlorophyllide a (Chlide a) and pheophorbide a (Pho a) or chlorophyllide b (Chlide b) and pheophorbide b (Pho b), respectively. These naturally occurring chlorophyll derivatives are abundant in green vegetables, but only a few studies have explored their chemopreventative properties [1–4].

In contrast, extensive studies have been done with Chlorophyllin (Chllin). Chllin is a commercially prepared, water-soluble, sodium–copper salt derivative of chlorophyll sold under the trade name Derifil. Chllin has been shown to be antimutagenic [5] and anticarcinogenic [6] when tested against various carcinogens. In particular Chllin has been shown to protect against the cancer-causing aflatoxins [7]. The mechanism of action has been demonstrated to involve trapping carcinogens via binding of the planar ring structures of the carcinogens to the planar ring structure in Chllin [8].

Aflatoxins are toxic metabolites produced by certain fungi. Aflatoxins regularly contaminate foods such as maize, peanuts, and fermented soybeans. The problem was first recognized in 1960 when

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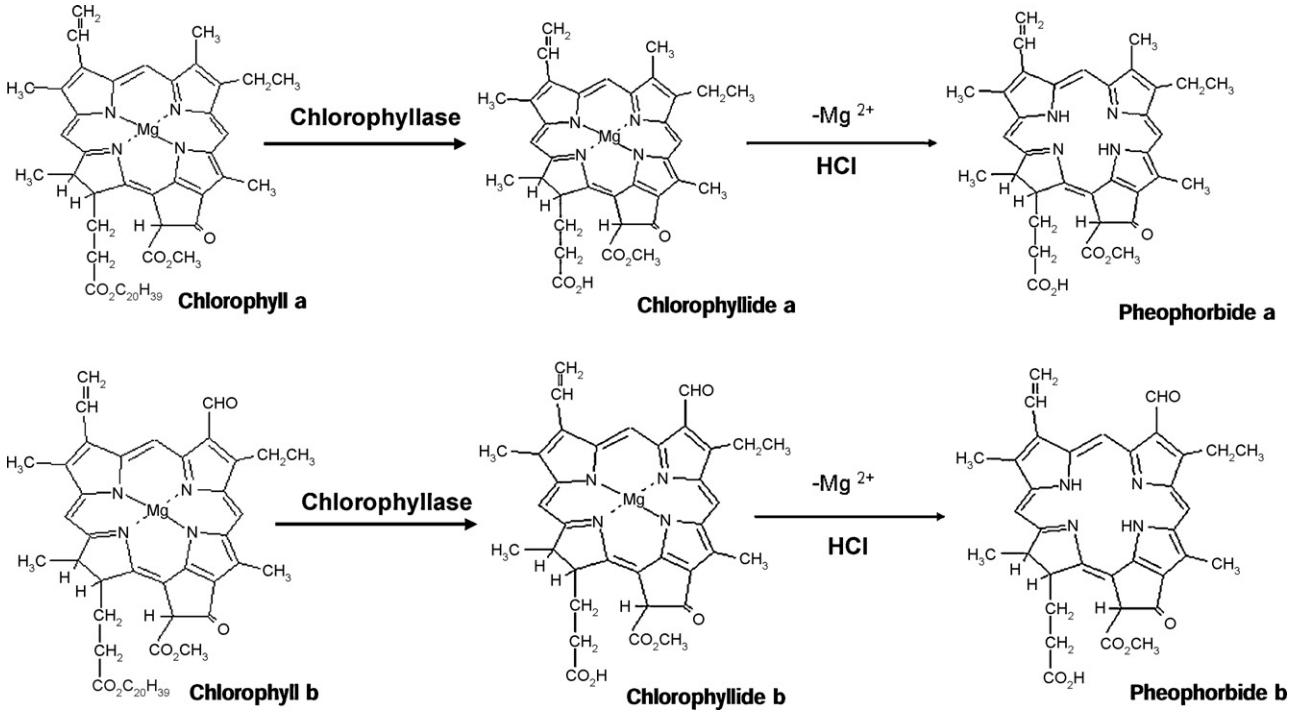

Fig. 1. Chlorophylls and their derivatives. Chlorophylls a and b are converted into their respective derivatives by dephytylation into chlorophyllide and subsequent magnesium dechelation into pheophorbide.

there was severe outbreak of a disease referred as "Turkey 'X' Disease" in the U.K., in which over 100,000 turkey poults died. Aflatoxin B_1 (AFB_1) is the most prevalent aflatoxin. Studies have shown that concurrent infection with the Hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC) in developing countries [9], particularly in Africa and Southeast Asia. Aflatoxin exposure and HBV infection are cosidered to be the two major risk factors for HCC. Although HBV can be controlled by vaccination, few interventions for aflatoxin exposure are available.

In animals, aflatoxin is metabolized through a number of competing pathways. In one pathway, AFB_1 has been shown to be activated by cytochrome P4503A4 (CYP3A4) into an epoxide, AFB_1 8,9-epoxide [10]. This highly reactive epoxide is known to form a covalent adduct with guanine yielding AFB_1-N^7-guanine (the major AFB_1-DNA adduct in the liver) [11]. Studies have shown that AFB_1-N^7-guanine causes a GC→TA transversion at a hotspot centered around codon 249 in the p53 gene [12], and that this p53-inactivating mutation is frequently found in HCC patients [13]. The adduct AFB_1-N^7-guanine is therefore used as a biomarker for aflatoxin poisoning, and it has been observed that elevated levels of this adduct are associated with an increased risk of liver cancer [7].

A major metabolic pathway detoxifying AFB_1 involves the glutathione *S*-transferase (GST) enzymes. GST enzymes conjugate AFB_1 8,9-epoxide with glutathione, preventing the epoxide from forming an adduct with DNA and facilitating the clearance of the bound epoxide from the body. In mice, the constitutive activity of alpha-class liver GSTs is high enough to protect mice from AFB_1 exposure, whereas in rats the constitutive GST activity is not great enough to protect them from AFB_1 exposure [14]. Experimentally feeding rats phytochemicals can boost hepatic GST activity and protect against AFB_1 exposure [15]. It has similarly been observed in primates that *Macaca fascicularis* has a naturally high GST activity against AFB_1, whereas humans do not [16].

In this study we challenged murine Hepa-1 hepatoma cell cultures with AFB_1 and measured the protective effects of the chlorophyll compounds Chllin, Chlide a, Chlide b, Pho a, and Pho b. AFB_1-DNA adduct formation was used as a measure. A wash-out experiment was used to see if the chlorophyll compounds produced their protective effect by conjugating with AFB_1. GST activity was measured to see if the protective effects correlated to an increase in GST activity. To our knowledge this is the first test of Chlide or Pho chemoprevention against AFB_1 toxicity. This study will help elucidate the basis of epidemiological observations of dietary cancer prevention in humans as well as explore the mechanism of action of these chlorophyll derivatives.

2. Materials and methods

2.1. Chlorophyll derivatives

Chlorophyll derivatives were prepared from spinach purchased in a local market in Taipei, Taiwan. They were prepared as previously described [17]. Briefly, chlorophylls a and b were extracted from the spinach was purchase from a local supplier, washed with cold water, and quickly freeze-dried the sample with liquid nitrogen and grind it into powder with pestle and stored at −70 °C until extraction. Grind and extract total pigment with 80% acetone; centrifuge the crude extract at 1500 × *g* for 5 min; and keep the supernatant and discard the pellet. Subsequently chlorophyll a and b were purified by liquid chromatography using a combination of ion-exchange and size exclusion chromatography with a CM-Sepharose CL-6B column. Analyses of chromatography fractions were performed by measuring the absorbance at 663.6 and 646.6 nm, which are the major absorption peaks of chlorophyll a and b. Chlorophyll a and b were dephytylated to Chlide a and b, respectively, by chlorophyllase isolated from the leaf of *Ficus macrocarpa*. The Chlide a and b were further Mg-dechelated to form Pho a and b by acidification with acetic acid, and allowed to stand for 2 min. All the samples were then dried under helium and stored at −70 °C for later use. Chlide and Pho, being more polar than chlorophyll, could be dissolved in phosphate-buffered saline (PBS) for use.

The purity of the chlorophyll derivatives was higher than 95%, as determined by reversed-phase high-performance liquid chromatography (RP-HPLC). Analytical separations were performed on a 5 μm Spherisorb ODS-2 column (25 cm × 0.4 cm, C_{18}). The chlorophyll derivatives were detected by fluorescence detection (excitation and emission wavelengths at 440 and 660 nm) and eluted using a linear gradient from solvent A (80% methanol in 1 M ammonium acetate) to solvent B (80% methanol in 1 M acetone) (Fig. 2) [18]. Chllin was from Sigma Chemical Co. (St. Louis, MO).

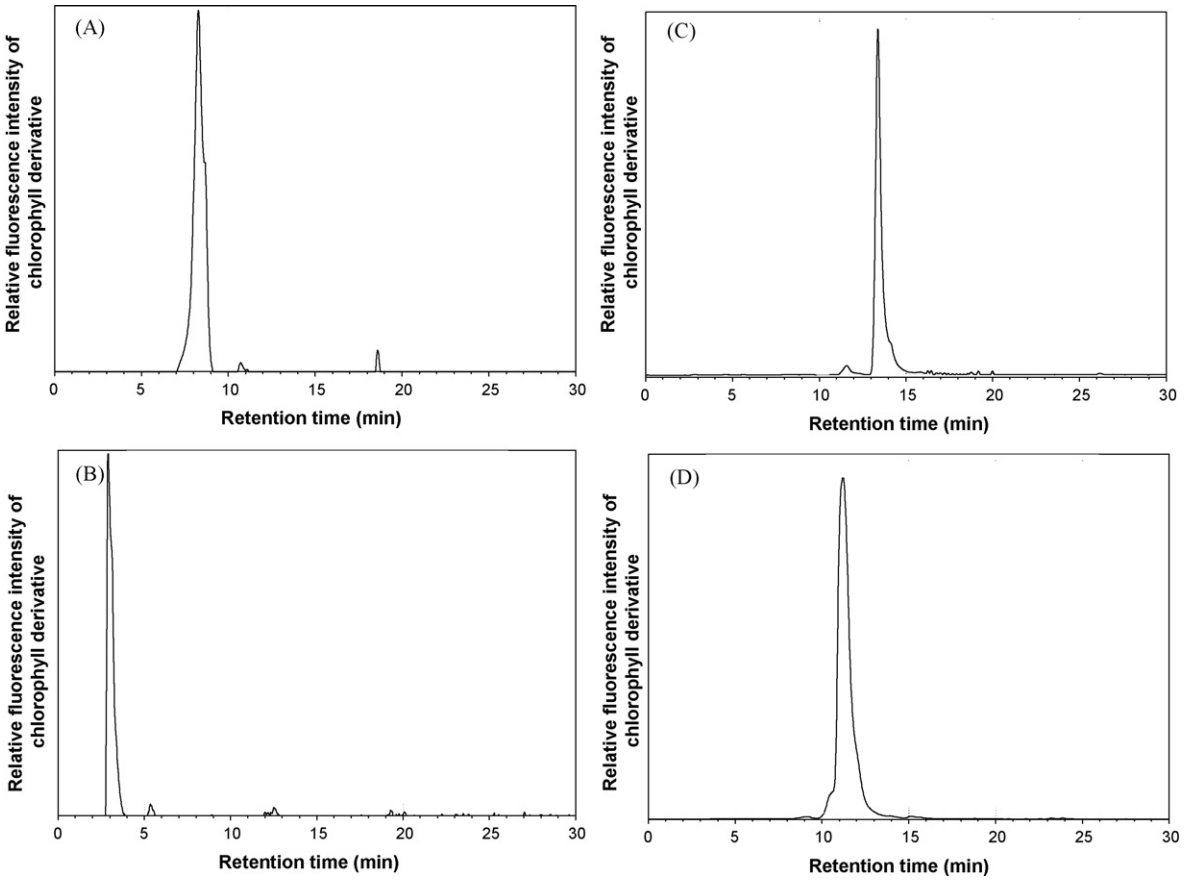

Fig. 2. Elution profile of the chlorophyll derivatives by RP-HPLC. Chlorophyll derivatives (chlorophyllide a (A); chlorophyllide b (B); pheophorbide a (C); and pheophorbide b (D)) were separated on a 5 μm Spherisorb ODS-2 column (25 cm × 0.4 cm, C_{18}), eluted with solvent A (80% methanol in 1 M ammonium acetate) and solvent B (80% methanol in 1 M acetone) by a linear gradient, and detected by a fluorescence detector (excitation and emission at 440 and 660 nm, respectively). All compounds prepared for this study showed greater than 95% purity.

2.2. Cell culture

In this study we used Hepa-1, an immortalized murine hepatoma cell line (Hepa-1c1c7) obtained from the Bioresource Collection and Research Centre (Taiwan). Although mice have been observed to be resistant to AFB_1 toxicity, the Hepa-1 cell line is highly sensitive to AFB_1 [19]. Cells were maintained by subculturing twice a week in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and incubating at 37 °C in a humidified atmosphere of 5% CO_2/95% air. For use in experiments, cells were harvested from continuous culture and adjusted to the appropriate cell density after counting on a hemocytometer.

In a control experiment to rule out cytotoxicity, Hepa-1 cells were cultured with the highest concentration of chlorophyll derivatives used (50 μM) and all the concentrations of AFB_1 used in later experiments. The media change protocol was the same as that used in AFB_1-DNA adducts analysis described below. Cell viability was determined at 96 h using an MTS assay kit (Promega, Madison, WI) following the manufacturer's instructions [20].

2.3. Analysis of aflatoxin B_1-DNA adducts

The effect of chlorophyll derivatives on AFB_1-DNA adduct formation was studied by treating Hepa-1 cells with different concentrations of AFB_1 and chlorophyll derivatives. Briefly, 1×10^6 Hepa-1 cells were cultured for 96 h with daily media changes. Cells received two PBS washes during each media change. The 0 and 24 h media changes contained different concentrations (0, 5, 20, or 50 μM) of chlorophyll derivatives (Chlide a, Chlide b, Pho a, Pho b, or Chllin). The 48 and 72 h media changes contained the chlorophyll derivatives and AFB_1 (0, 5, or 10 ng/ml). The washout variation of this experiment used media containing only AFB_1 during the 48 and 72 h media changes. In order to measure DNA damage after AFB_1 treatment, DNA was purified at 96 h from treated Hepa-1 cells using a PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

The levels of AFB_1-DNA adducts were then measured by competitive ELISA using antibody 6A10 as described previously [21]. Briefly, the ELISA used Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) coated with 5 ng of imidazole ring-opened AFB_1-DNA in PBS by drying overnight at 37 °C. The test solutions contained unbound AFB_1-DNA and antibody. Goat anti-mouse IgG alkaline phosphatase (1:1500) and then *p*-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 8.6) was added to the DNA. After 90 min incubation at 37 °C, absorbance at 405 nm was read on a Bio-Tek microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

For the test samples, 25 μg denatured Hepa-1 DNA in 50 μl hydration solution was mixed with 50 μl diluted antibody before being added to the wells. The level of AFB_1-DNA in the test samples was quantitated relative to a standard curve based on known concentrations of AFB_1-DNA.

For the standard curve, highly modified imidazole ring-opened AFB_1-DNA was serially diluted with nonmodified denatured calf thymus DNA such that 50 μl contained from 0 to 1000 fmol adduct and 50 μg DNA. These samples were mixed with an equal volume of diluted 6A10 antibody (50 μl, diluted $1{:}1.25 \times 10^6$), added to the wells, and measured by competitive ELISA.

2.4. Analysis of glutathione S-transferase (GST) activity

GST activity was measured with a GST assay kit (Calbiochem, Bad Soden, Germany) using glutathione (GSH) and 1-chloro-2,4-dinitrobenze (CDNB) as substrates [22]. Briefly, CDNB–GSH conjugate formation (GST activity) was recorded continuously in potassium phosphate buffer (pH 6.5) containing cell lysates, GSH, and CNDB using a spectrophotometer set at 340 nm. The slopes (ΔA_{340}/min) of the sample and blank were recorded over a 5-min period, and the extinction coefficient of the CDNB–GSH conjugate was 9.6 mM^{-1} cm^{-1}. The activity was expressed as Δ concentration of CDNB–GSH conjugate/mg protein.

2.5. Statistical analysis

All values were expressed as means ± S.D. Data were analyzed using SPSS software (Version 10.0). Statistical significance was determined using one way ANOVA followed by Scheffe's *post hoc* multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity

We first tested our experimental system to see if any of the components caused cytotoxicity when measured by MTS assay. Treatment of Hepa-1 cells with the maximum dose (50 μM) of chlorophyll derivatives alone showed no significant decrease in cell survival. Likewise, when treated with either AFB_1 alone or in combination with chlorophyll derivatives, no significant decrease in cell survival was observed.

3.2. Effect of chlorophyll derivatives on formation of AFB_1-DNA adducts in Hepa-1 cells

As a biomarker for mutagenicity, AFB_1-DNA adducts were measured in a competitive ELISA with a monoclonal antibody designed to specifically bind AFB_1-DNA adducts [21]. Fig. 3A shows the amount of AFB_1-DNA adduct formed after treatment with 5 ng/ml AFB_1 in the presence of 0–50 μM of each chlorophyll derivative. All chlorophyll derivatives increasingly inhibited AFB_1-DNA adduct formation as the dose was increased. At high concentrations, all chlorophyll derivatives showed statistically significant inhibition. Pho a and b produced the greatest inhibition among the chlorophyll derivatives.

Fig. 3B shows the same experiment as above using 10 ng/ml AFB_1. Note that the baseline with 0 μM of each chlorophyll derivative has a higher amount of AFB_1-DNA adduct formation than was seen with 5 ng/ml AFB_1, consistent with an AFB_1-dose-dependent formation of adducts. Again, increasing concentrations of chlorophyll derivatives showed increasing and statistically significant inhibition of AFB_1-DNA adduct formation with Pho a and Pho b among the most potent inhibitors at high concentration.

3.3. Effect of wash-out on formation of AFB_1-DNA adducts in Hepa-1 cells

All or part of the inhibition of formation of AFB_1-DNA adducts in Hepa-1 cells seen after chlorophyll derivatives treatment may be due to direct binding of AFB1 by the derivatives, thus preventing AFB_1 from being metabolized into AFB_1 8,9-epoxide and forming DNA adducts. In order to address this possibility, a wash-out experiment was performed in which the cells were pretreated

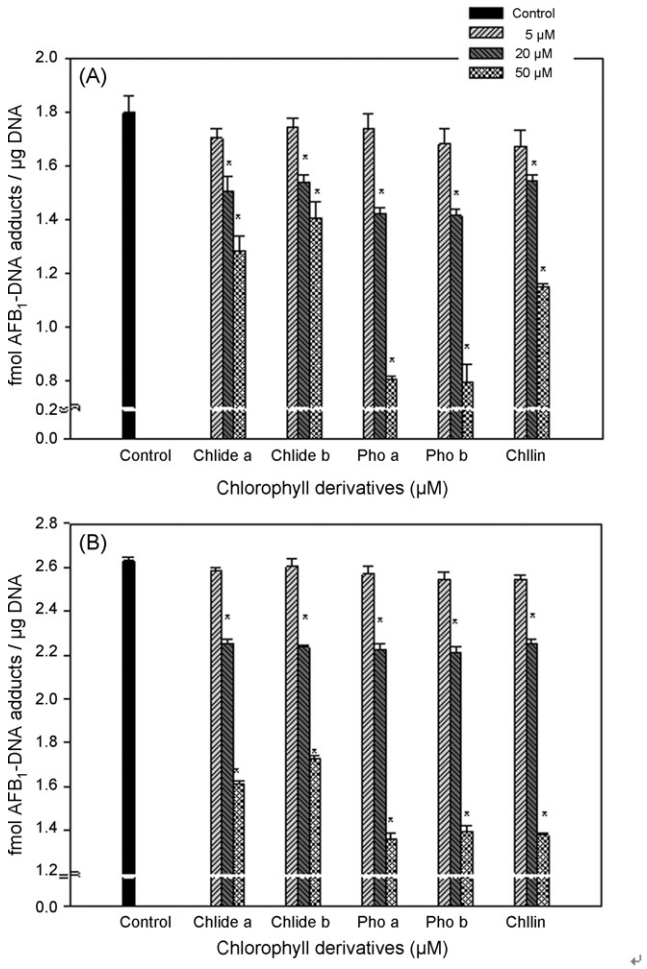

Fig. 3. Effect of pretreatment with chlorophyll derivatives before challenge with 5 ng/ml (A) or 10 ng/ml (B) AFB_1. AFB_1-DNA adduct formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a biomarker for AFB_1 carcinogenic potential. Results are expressed as the mean ± S.D. Note that histograms (A) and (B) do not use the same scale *y*-axis. (*) Significant difference ($P < 0.05$) from control value.

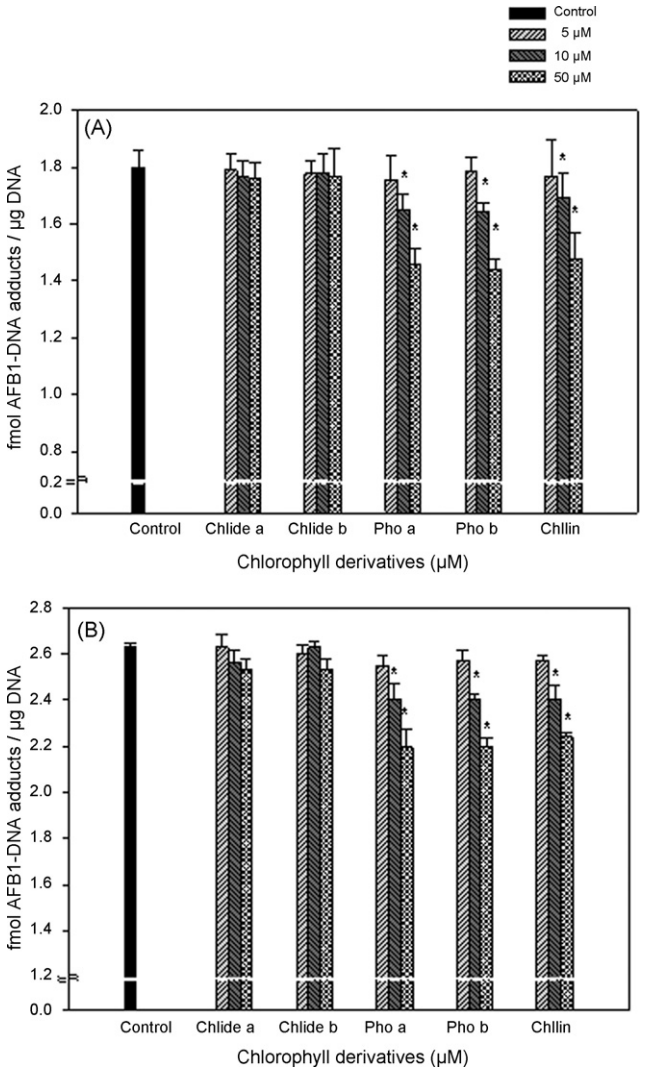

Fig. 4. Effect of pretreatment with followed by wash-out of chlorophyll derivatives before challenge with 5 ng/ml (A) or 10 ng/ml (B) AFB_1. AFB_1-DNA adduct formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a proxy for AFB_1 carcinogenic potential. Results are expressed as the mean ± S.D. Note that histograms (A) and (B) do not use the same scale *y*-axis. (*) Significant difference ($P < 0.05$) from control value.

with chlorophyll derivatives during the first two media changes, but only AFB_1 and no chlorophyll derivatives were present in last two the media changes. We reasoned that if the chlorophyll derivatives inhibited AFB_1 adduct formation by directly binding the AFB_1, then there should be no inhibition of AFB_1 if there was no chlorophyll derivate present in the media during treatment with AFB_1.

Fig. 4A shows the results of the wash-out experiments with 5 ng/ml AFB_1. Compare Fig. 4A to the parallel experiment in Fig. 3A and note that in the cultures receiving no chlorophyll derivative treatments (0 μM), the level of AFB_1-DNA adduct formation is the same; this indicates that the two experimental data sets are in agreement. All chlorophyll derivatives at all concentrations inhibited AFB_1-DNA adduct formation less in the wash-out experiment. Most dramatically, Chlide a and b no longer inhibited AFB_1-DNA adduct formation in the wash-out experiment. Pho a, Pho b and Chllin all had reduced inhibition of AFB_1-DNA adduct formation in the wash-out experiment, but could still significantly inhibit adduct formation. Fig. 4B shows similar results when using a concentration of 10 ng/ml AFB_1.

3.4. Effect of chlorophyll derivatives on GST activity in Hepa-1 cells

To explore the possibility of an indirect protective effect of chlorophyll derivatives on AFB_1-induced DNA damage, the effect of chlorophyll derivatives on GST activity in AFB_1-treated Hepa-1 cells was studied (Fig. 5). Chlide a and b did not show any statistically significant difference in GST activity in AFB_1-treated (at either 5 or 10 ng/ml) Hepa-1 cells. Pho a, Pho b and Chllin treatment had significant dose-dependent increases in GST activity.

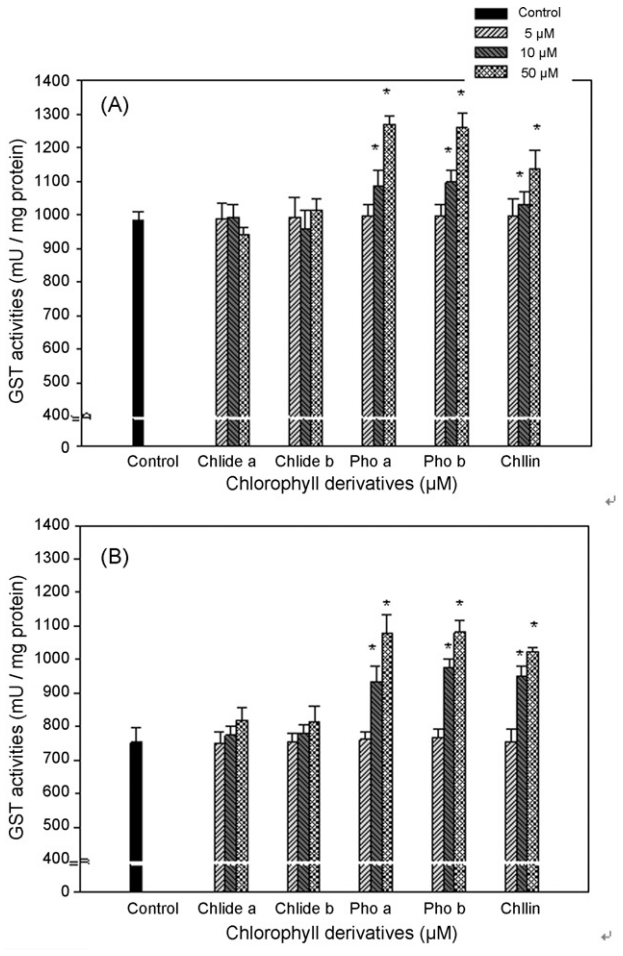

Fig. 5. Effect of chlorophyll derivatives on GST activity in AFB_1-induced Hepa-1 cells. Cells were first treated with various derivatives for 48 h and then treated with 5 ng/ml (A) or 10 ng/ml (B) AFB_1 for another 48 h. GST activity was evaluated using a commercial kit. Results are expressed as the mean ± S.D. (*) Significant difference ($P < 0.05$) from control value.

4. Discussion

In this study we looked at the protective effects of chlorophyll derivatives against AFB_1-DNA adduct formation. We found a significant inhibition of AFB_1-DNA adduct formation. We further showed that the wash-out experiment partially or completely eliminated this inhibitory effect. Finally, we examined the effects of the test compounds on GST activation and found that Pho and Chllin could significantly increase GST activity.

Pho a can be used to kill cells through photodynamic therapy, where a light source such as a laser is used to stimulate free radical production by Pho a, ultimately leading to growth arrest and apoptosis [23,24]. Because chlorophyll compounds can become cytotoxic in certain situations, we first tested for cytotoxicity and found that none of the treatments used in the study caused cell death.

Levels of AFB_1-DNA adducts have been positively associated with risk of liver cancer and have been used as a biomarker of AFB_1 exposure [25]. By measuring AFB_1-DNA adduct formation we were able to observe protection against adduct formation by chlorophyll derivatives in a dose-dependent manner. The degree of protection was greatest for Pho and least for Chlide.

Numerous *in vitro* studies have indicated that chlorophyll derivatives attenuate chemical genotoxicity by forming a molecular complex with promutagens [8,26–28], which may involve strong chlorophyll–AFB_1 interaction *via* their planar unsaturated cyclic rings [27]. The complete elimination of the Chlide inhibitory effect after the wash-out experiment shows that the effects of Chlide occur in the culture media and not in the cells. The best explanation for its activity is that Chlide is directly binding to and neutralizing AFB_1. The fact that the effects of Chlide are dose-responsive also supports such a conclusion.

That Chllin and Pho also showed a reduction of ability to inhibit AFB_1-DNA adduct formation in the wash-out experiment means that part of their effects are related to their presence in the cell culture media. The simple explanation that Chllin and Pho also bind and neutralize AFB_1 is reasonable. That Chllin and Pho retained activity after wash-out was surprising. Hypothesizing that some Pho may have been absorbed into the cells, we performed a control experiment (data not shown) in which Hepa-1 cells were treated with the test compounds, Chlide a and b and Pho a and b, for 48 h then thoroughly washed free of medium and tested for presence of these chlorophyll derivatives in the cells by HPLC analysis of ultrasonicated cell lysates; Pho a and b could be found in the cell lysates whereas very little Chlide a and b could be found. Thus, in the wash-out experiment, Pho was retained between media changes sequestered in the cells and was available to bind to AFB_1 entering the cells when the cells were challenged with AFB_1

However, it is also possible that, in addition to directly binding AFB_1, Chllin and Pho (but not Chlide) interact with and change the Hepa-1 cells, increasing cellular resistance to AFB_1-DNA adduct formation. Thus, part of the protection provided by Chllin and Pho would have been via direct binding of AFB_1 and part of the protection would have been provided by stimulation of cellular defenses. Such a hypothesis also fits the wash-out data; after the pretreatment media containing Chllin or Pho was removed and the AFB_1-containing media was added, the cells could still have been resistant to AFB_1 because the cells themselves had become resistant to AFB_1 due to a dose-dependent stimulation of cellular defenses.

This mechanistic hypothesis is attractive because it would also explain why there is a difference in the magnitude of the protective inhibitory effect of Chlide versus Pho or Chllin. Because the chlorophyll derivatives have similar molecular structures and were present in the same molarities, a simple binding reaction would be expected to remove and inactivate similar quantities of AFB_1. The larger inhibitory effects of Pho and Chllin could be the additive result of their stimulation of cellular defenses plus their AFB_1 binding. If their putative cell stimulatory effects were blocked and only their ability to bind AFB_1 remained, we would expect that the degree of inhibition might be close to that of Chlide.

In order to look for stimulation of cellular defenses, we measured the ability of the chlorophyll derivatives to increase GST activity. Chllin and Pho were able to significantly increase GST activity in a dose-dependent manner in Hepa-1 cells, while Chlide could not. Thus, it seems reasonable to conclude that the inhibition of AFB_1-DNA adduct formation by Chlide a and Chlide b was due to direct molecular trapping of AFB_1, and that the greater inhibition of AFB_1-DNA adduct formation by Chllin, Pho a and Pho b was due to both direct molecular trapping of AFB_1 and stimulation of cellular defenses.

Little has been published about the absorption of chlorophyll derivatives into cells, however, some data is available about the absorption of Pho a. In general, it appears that passive diffusion of Pho a into cells is counteracted by active transport of Pho a out of cells by ATP-binding cassette (ABC) transporters such as the breast cancer resistance protein (ABCG2) [29]. Tumor cells have been noted to preferentially absorb Pho a compared to normal cells [30–32] and to concentrate Pho a in the mitochondria [33,34]. In contrast, drug-resistant cancer cell lines typically have increased expression of the ABC transporters. AFB_1 penetrance into cells is similarly affected by ABC transporters [35]. The fact that the Hepa-1 cell line used in this experiment is highly sensitive to AFB_1 suggests that Hepa-1 cells have low ABC transporter expression. This agrees with our data showing that the Hepa-1 cells absorbed Pho. Whether or not any absorbed Pho or Chllin would have passively diffused out of the cells and diluted into the fresh AFB_1-containing medium during the washout experiment remains to be investigated.

Fahey et al. [36] directly tested Chllin against murine hepatoma cells *in vitro* and showed that Chllin induces the phase II enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1). Likewise, Singh et al. [37] showed that short-term oral administration of Chllin increases the levels of hepatic GST in lactating mice and suckling pups. These *in vivo* and *in vitro* results parallel our *in vitro* results. In contrast Simonich et al. [38] also tested the ability of Chllin and chlorophyll to prevent AFB_1 toxicity *in vivo*. Rats given dietary Chllin and chlorophyll and then challenged with AFB_1 have reduced AFB_1-adduct formation, but do not show a significant effect on the phase II enzymes GST and NQO1 [42]. Thus, the *in vitro* results presented here need to be weighed against the possibility that much of the dietary chlorophyll compounds are kept from entering the body by ABC transporters in the intestines and that the majority of chlorophyll compounds' interaction with dietary AFB_1 *in vivo* may take place in the intestinal lumen through direct trapping.

In conclusion, we have provided evidence that chlorophyll derivatives can reduce AFB_1-DNA adduct formation *in vitro*. Diets rich in chlorophyll may prevent the development of hepatocellular carcinoma. We also provide evidence that the mechanisms and intensity of protective effects may be dependent on the particular chlorophyll derivative used.

5. Conflict of interest

None.

Acknowledgments

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