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GROWTH INHIBITORY EFFECT OF CHLOROPHYLLS IN CULTURED U87 GLIOBLASTOMA CELLS

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ABSTRACT: ***We employed glioblastoma (human-derived U87 cells) cells as an experimental model for our present investigation. Chlorophyll a and b, purified from Spinacia Oleracea, were incubated together with glioblastoma cells at concentrations ranging from 10 nM to 1 μM. Assessment of growth was determined by cell counting by using a hemocytometer or by WST-1 assay. Changes in survival signals (Akt, ERK, and p-ERK), apoptosis (Bax, caspase 3, and p21), levels of cell cycle cdk1/cdc2 protein and PTEN/ p-PTEN were observed and Hoescht 33342 nuclear staining was also performed. Box plotting and Student t test was used for statistical analysis. It was observed that at concentrations higher than 10 nM, there was a decline in the rate of cell proliferation. At a concentration of 100 nM, proliferation of cells stopped with decreased phosphorylation of PTEN. Whereas, there were no changes in other cell survival related proteins. Chromatin condensation or nuclear fragmentation was not detected by Hoescht 33342 staining. Combination of chlorophylls a and b neither exhibited an additive nor any synergistic effect on the cells. In conclusion, chlorophylls inhibited the growth of cultured U87 glioblastoma cells, indicating its function as a cytostatic agent.***

KEY WORDS: Chlorophyll, Glioblastoma, Proliferation, PTEN

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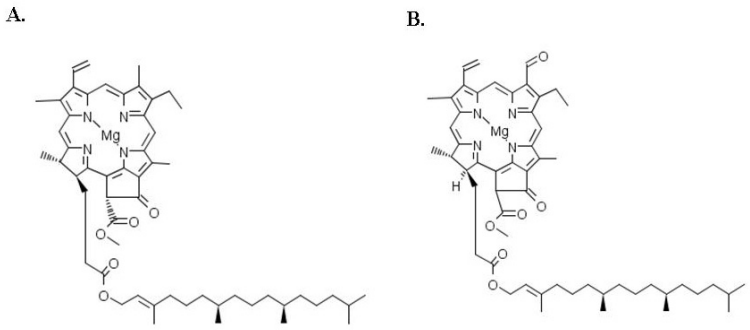
INTRODUCTION

Vegetable and dietary therapies are previously applied in advanced phases of cancer (Donaldson, 2004). Green vegetable juice is composed of fibers, vitamins, enzymes, and other nutritional elements. Chlorophylls are not only the green pigment of plant cells, but also a crucial requirement for the energy processes of life. Chlorophylls are located in the chloroplast in the form of chlorophyll a and b, in a ratio of 3:1 or 3:2, respectively. Both the types are composed of a porphyrin ring, which interacts with magnesium in the form of a chelate (Figure 1) (I., 1967).

FIGURE 1. Structure of chlorophyll a and b isolated from *Spinacia Oleracea*. The figure illustrates the presence of phorphyrin-like structure with magnesium chelated at the center. **(A)**. Chlorophyll a, **(B)**. Chlorophyll b. [The drawing of the structures was provided by KegDraw program (www.genome.jp)]



Several reports are available on the effects of chlorophyll on the human body. Chlorophyll is similar in structure to hemoglobin, where Mg is present in the center instead of Fe; hence the role of cholorphyll in hematogenesis has been proposed. When chlorophyll is absorbed in the intestine, it is changed to heme, and then gets transferred into the blood cells (Granick and Beale, 1978; Hawkins. et al., 1986). The heme groups are involved in catalase activity, which catalyzes per-oxidation, indicating imperative role in oxidative stress. The heme groups are also known to enhance liver function, in

detoxification and assisting in export of toxic materials (Egner et al., 2001). Chlorophyll is also known to possess an anti-cancer effect. It reduces the carcinogenic activity, thus preventing the occurrence of cancer (Chernomorsky et al., 1999; Sarkar et al., 1994). However, the direct effect of chlorophylls on the growth of cancerous cells has rarely been studied (Balder et al., 2006; de Vogel et al., 2005; Park et al., 1989).

In our present study, we have attempted to determine whether chlorophyll can affect the growth of Glioblastoma Multiforme (GM). Cells originated from human glioblastoma were cultured with chlorophylls and assessed for cell growth *in vitro*.

MATERIAL AND METHODS

Preparation of Chlorophylls a and b

Chlorophylls a and b, whose structures are shown in Figure 1 (Chlorophyll a: catalog number c5753, $C_{55}H_{72}MgN_4O_5$, MW 893.49/ Chlorophyll b: catalog number c5878, $C_{55}H_{70}MgN_4O_{6,}$ MW 907.46), were derived from spinach (*Spinacia Oleracea*) leaves (Sigma, St Louis, MO, USA). The purities were e"90.0% (HPLC) for both the chlorophylls (Ruhle and Paulsen, 2011). They were initially dissolved in 10 ul of ethanol and were incubated together with culture media at doses of 10 nM, 100 nM, and 1 μM. The control (solvent only) was tested under identical conditions.

U87 Glioblastoma Cell Culture and Chlorophyll Treatment

Human glioblastoma U87 cells (American Tissue Culture Collection) were used as an experimental model of GM (Lin et al., 2007a; Lin et al., 2007b). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin (Sigma, St Louis, MO, USA), and L-glutamine (2 mM) at 37°C in 5% CO_2 and 95% air. Chlorophylls a and b were incubated together with culture media at doses of 10 nM, 100 nM, and 1 μM. The control (solvent only) was tested under identical conditions. Twenty four-well plates were used for cell counting, and 96-well plates were used for the WST-1 assay. We used 100 mm cell culture plates for western blotting. For Hoescht 33342 nuclear staining, cells were plated on poly-L-lysine treated cover slips. Cells were maintained for 24 or 48 hours.

As a positive control, triptolide ($C_{20}H_{24}O_{6,}$ MW 360.40) was employed as a reference compound, which is known as a growth inhibitor for U87 glioblastoma cells (Lin et al., 2007b). The compound is a diterpene triepoxide, the principal active ingredient, originated from extracts of the Chinese herb, *Tripterygium wilfordii* hook. Triptolide is also commercially available from Sigma Aldrich with purity of e"98% (HPLC). The compound has been reported to inhibit the growth of U87 glioblastoma cells in a dose-dependent manner; 92 ± 9.5 % at 1 ng/mL (mean ± SD), 75 ± 7.5 % at 5 ng/mL, 49.5 ± 4.5 % at 20 ng/mL, and 4.4 ± 0.7 % at 50 ng/mL, percentage relative to control (100 ± 8.5 % at 0 ng/mL) (Lin et al., 2007b).

WST-1 assay

For comparison of differences in absorption (ABS) between control and chlorophyll treated groups, cells were cultured in 96-well plates. The initial cell incubation required a volume of 200 μL/well of culture medium (Falcon; Becton Dickinson, Lincoln Park, NY, USA), and a WST-1 reagent (20 μL/well). Later, the cells were cultured at a volume of 100 μL/well and 10 μL/well of WST-1 (final dilution 1:10 in each case) was added. Subsequently, in order to determine the optimal incubation period, we measured absorption at multiple time points after addition of WST-1 (e.g., 0.5, 1, 2, and 4 hours after the initial setup). For those series requiring higher sensitivity, we increased the incubation time (half maximum absorbance (ABS) after incubation with WST-1 reagent solution for 0.5 hours with 2000 cells/well or for 4 hours with 7000 cells/well). Addition of WST 1 to the culture medium in the absence of cells resulted in the occurrence of spontaneous absorbance value. As the background ABS was dependent on the culture medium, incubation time, and exposure to light; for the background control (blank), similar volume of culture medium and WST-1 was added to one well of each plate and used this well (which, in the absence of cells, had an absorbance equal to culture medium + WST 1 + 1 μM chlorophyll) for the reader's blank position as "0". The absorbance was measured at 450 nm, and the measurements were performed in triplicate. For determination of data by WST-1 assay, a comparison of ABS values was carried out between each well with chlorophylls and the control wells. Box-plots were used for analysis and distribution of WST-1 data.

Cell Counting

U87 cells were cultured on a 24-well culture plate, and, to ensure the presence of similar number of cells in each well, cells were equally diluted and cell counting was performed in initial concentration by using a hemocytometer. After removal of DMEM media from the wells, the plates were washed with phosphate-buffered saline (PBS), followed by addition of 0.5 mL of 0.5% tris-EDTA (TE) buffer. The plates were then placed in the incubator at 37! for three minutes. To inhibit the effect of trypsin, 0.5 mL of DMEM media was also added. The solution was mixed, for at least ten times by pipetting, to ensure uniformity in the cell density. A hemocytometer (Marienfeld, U.S) was used for cell counting. The volume of one 5 x 5 square equals to 0.1 mm^3 (1 mm ×1 mm ×0.1 mm), which is equivalent to 10^{-4} mL. Thus, the

density of cells in the solution was (number of cells) x 10^4/mL. At least three trials were performed for each cell counting. We confirmed that WST-1 assay values were in correlation with the number of cell counts in a dose-dependent manner.

Hoechst 33342 Nuclear Staining

Hoechst 33342 staining followed by fluorescence microscopy can reveal the presence of a cell undergoing apoptosis, in terms of nuclear condensation and fragmentation. We prepared the Hoechst solution, coated each culture dish with poly-L-lysine, and washed the cells with PBS for five minutes. U87 cells that had been incubated, as described above, were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (5 mg/mL) for 20 minutes at room temperature. After fixation and staining, cells were incubated at room temperature (25 ^{0}C) for five minutes and washed in PBS, three times at intervals of 10 min. Finally, we added the mounting solution (Dako, Glostrup, Denmark) and dried the cells for later evaluation. Positive control was also treated in the similar manner to determine the apoptotic features in the glioblastoma cells.

Western Blotting

Cells were collected by using a cell scraper. Cellular protein was extracted by homogenizing the cells with mammalian protein extraction reagent (M-PER) cell-lysis solution (Molecular Probes, Eugene, OR, USA) by using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA, USA). Protein extracts from the cells (20µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by using 10% polyacrylamide with 0.05% bis-acrylamide, according to published procedures (DiFiglia et al., 1995). The separated proteins were then transferred to nitrocellulose membrane, and the blots were probed with antibodies specific for survival signals (Akt, ERK/p-ERK); apoptotic or proapoptotic proteins (Bax, Caspase 3, and p21), cell cycle cdk1/cdc2 proteins, and PTEN/p-PTEN (phosphatase and tension homologue). Primary antibodies against ERK, p-ERK, PTEN, p-PTEN, and caspase3 were purchased from Cell Signaling Technology (Beverly, MA). Anti-Akt (SC-5298), anti-Bax (SC-526), and anti-p21 (SC-397) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and anti-cdk1 and anti-cdc2 were from Millipore Corp (Bedford, MA). Anti-ß-actin antibody (Sigma Aldrich, 1:5000 dilution, monoclonal) was used as an internal control. The positive signals for each antibody were confirmed by the provided information or based on the experimental condition from references. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Vector laboratory, CA, USA) was used as a secondary antibody with 1:3000 dilution. Immunoreactivities were detected by ECL (enhanced chemiluminescence) solution (Supersignal, Pierce, Rockford, IN). Film was exposed for 10 to 30 minutes.

Data Analysis

Box plotting was used for the determination of data scattering, data efficiency, and other statistical properties, depicting groups of numerical data through their five-number summaries (the smallest observation [L], lowest quartile [Q1], median [Q2], upper quartile [Q3], and largest observation [H]). For this type of statistical analysis, the value L was calculated as Q1 " (1.5 × (Q3 " Q1)), and the value H was calculated as Q3 + (1.5 × (Q3 " Q1)). L and H values revealed 99% area regions of the T-distribution plots, indicating that it was appropriate to regard the data that fall outside these ranges as outliers. With these box plots, we used the Student's t-test for analysis of data.

RESULTS

Cell Line Property Determination: Glioblastoma cells revealed a doubling time of 24 hours. When cells were plated at approximately 50% confluency, they became 100% confluent at 24 hours point time. In this experiment, they were initially plated at 25% confluency. Culture plate showed 50% confluency with cancerous cells at 24 hours. At 48 hours, the cancerous cells became almost confluent and thereafter, they became compact due to the presence of other cells and inhibiting further expansion on their own. Therefore, the appropriate time point for assessment of the effect of chlorophylls was determined to be between the 24 and 48 hours point.

Cell Proliferation Assay: For WST-1 assay, cells were treated with chlorophyll a or chlorophyll b at a concentration of 1ìM, and were analyzed at 24 hours. Chlorophyll a or b treatment resulted in lowered ABS values by spectrophotometric measurement ($P < 0.05$, t-test). Incubation of both, chlorophyll a (1 ìM) and b (1 ìM) together also resulted in a decreased ABS value (Figure 2A). However, the mean and median values were not different from the independent measurement in the presence of chlorophyll a or b ($P > 0.05$) (Figure 2B). To further determine the growth inhibitory effect of chlorophylls, cells were treated at lower concentrations, and counted. At a concentration of 100 nM, there was a decrease in the number of cells, 52.0 $\pm$ 5.3 % in chlorophyll a and 60 $\pm$ 6.3 % in chlorophyll b at 24 hours ($P < 0.05$ by Student t-test)(Figure 3A). In addition, further increase in cell numbers was not observed at 100 nM between 24 and 48 hours. Also, there was no difference in the morphology of the cells (Figure 3B). At a concentration of 10 nM, the number of cells was doubled between 24 and 48 hours (Figure 3A). The control group also showed the expansion in cell number,

which was doubled between 24 and 48 hours, indicating that 10 nM group was not significantly different from that of control group.

FIGURE 2. WST-1 assay for chlorophylls treatment. Cells were incubated with chlorophyll a, b, or both at a concentration of 1 µM for 24 hours. (**A**). Mean values for chlorophylls treatment were lower than that of the control (mean ± standard deviation; *, #, P < 0.05, n = 10, t-test). When chlorophylls a and b were used together, ABS value was not different from the values of independent treatments with chlorophyll a or chlorophyll b (P > 0.05). The ABS value was set to "0" for the blank cuvette with the medium and 1 µM of chlorophyll. (**B**). Box plot of WST-1 data distribution. All the measured values were within the range of normal distribution, indicating that the groups can be statistically compared by Student t-test. (ABS: absorbance, C: control group without chlorophyll treatment, A: chlorophyll a, B: chlorophyll b, A+B or AB: both chlorophyll a and b)

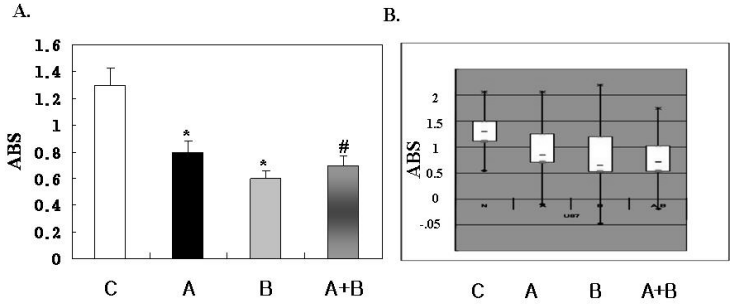


FIGURE 3. Cell counting data and growth of U87 glioblastoma cells. (**A**). Cells were incubated with chlorophyll a or b at the concentration of 10 nM and 100 nM for 24 hours. Mean values at 100 nM, were lower than that of control in the presence of both chlorophyll a and b (mean ± standard deviation, * p < 0.05, n = 10, t-test). However, at 10 nM, there was no significant difference in the number of cells, when compared with the control. [White column (control), gray column (10 nM), black column (100 nM), X axis-concentration, Y axis-Relative ratio to control]. (**B**). Under control conditions (without chlorophylls), glioblastoma cells exhibited growth proliferation. At the 48 hours point, the number of cells increased approximately by two-fold when compared to 24 hours. However, when the cells were incubated with 100 nM of chlorophyll a or b, they did not reveal any increases in numbers and of the cell number remained constant like 24 hours data. Dark spots are chlorophyll b pigments (bar = 50 µm).

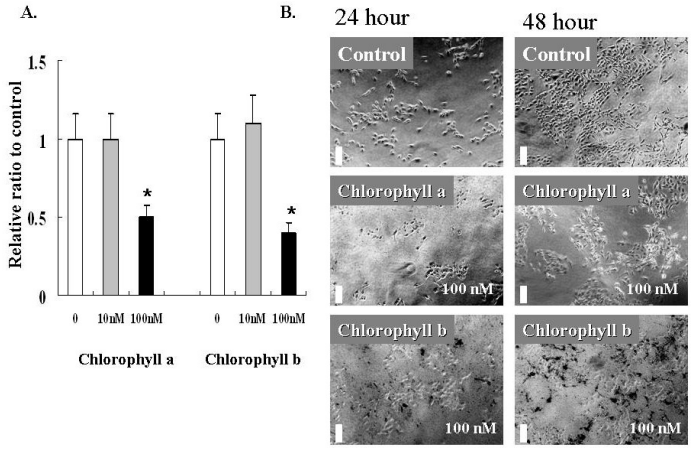
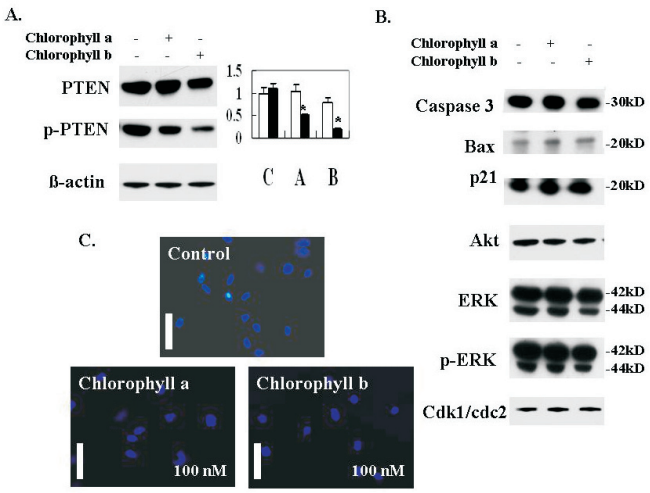


FIGURE 4. Immunoblots and Hoescht 33342 nuclear staining. Cells were incubated with 100 nM chlorophyll for 24 hours. (**A**). The immunoreactivities of PTEN protein was detected at 54 kD. Treatment with chlorophyll a or b decreased the phosphorylated form of PTEN (White column-optical density of PTEN; Black column:optical density of p-PTEN; X-axis C: control, A: chlorophyll a, B: chlorophyll b: A+B: both chlorophylls; Y-axis: relative optical density to ß actin, each columns indicates mean ± S.D, * P < 0.05 by Mann-Whitney non-parametric test, n = 6) (**B**). In the case of survival signals, Akt, ERK and p-ERK remained unchanged. Protein levels of bax, p21, and caspase 3 did not revealed any changes. The levels of Cdk1/cdc2 cell cycle proteins were not altered. **C**. Hoescht 33342 nuclear staining did not exhibited any nuclear fragmentation or chromatin condensation, although there was a decrease in cell number (bar = 50 µm).



Cell Proliferation Assay: At the concentration of 100 nM, when cells did not exhibited further proliferation, we determined whether the cells showed alteration in apoptosis, survival pathway, or cell cycle signals. PTEN (phosphatase and tension homologue), which has been reported to result in cell cycle arrest in glioma cell lines (Cheney et al., 1998; Furnari et al., 1998; Li and Sun, 1998), exhibited a decrease in the phosphorylated form in the presence of chlorophylls a and b (Figure 4A). Akt or ERK/p-ERK remained unchanged (Figure 4B). Apoptotic protein levels of bax, p21, and caspase 3 also did not showed any alteration (Figure 4B). Also, there were no changes in the levels of Cdk1/cdc2 cell cycle proteins. Hoescht 33382 nuclear staining did not exhibited any nuclear fragmentation or chromatin condensation (Figure 4C).

DISCUSSION

The purpose of this study was to determine whether chlorophylls possessed any inhibitory effect on a cellular

model of glioblastoma. Based on the experiments, it was confirmed that proliferation of U87 glioblastoma cells was inhibited in the presence of chlorophylls. Arrest in the growth of the glioblastoma cells was associated with decreased phosphorylation of PTEN. Under this condition, neither there was any alteration of akt, ERK, or cdk1/cdc2 cell cycle proteins, nor any evidence of apoptosis in relation to levels of bax, caspase 3, p21, and Hoescht nuclear staining was detected. Combination of chlorophylls a and b did not revealed any additive or synergistic growth inhibitory effect, as determined by the WST-1 assay at 1 μM concentration.

In our experiment, presence or absence of apoptosis could not account for the inhibition of glioblastoma cell growth by chlorophylls. There was no alteration in the expression of bax, caspase 3, and p21, and Hoescht nuclear staining did not showed any morphological changes, which are characteristics of apoptosis. As for the changes in cell cycle, there was no change in the number of cells between 24 and 48 hours at a concentration of 100 nM. This observation further revealed that there was arrest in cell proliferation. However, there was no variation in the protein levels of cdk1/cdc2 in our experiment, suggesting that inhibition of U87 cell growth is not mediated by these proteins. Instead, PTEN, which is involved in cell cycle arrest of glioblstoma cells (Cheney et al., 1998; Furnari et al., 1998; Li and Sun, 1998), showed decreased in phosphorylation, suggesting that chlorophyll can inhibit proliferation of glioblastoma cells, associated with phosphorylation of PTEN protein.

WST-1 assay showed that both the types of chlorophylls were effective in inhibiting growth of U87 glioblastoma cells. As further proliferation was not observed at 100 nm concentration or more, we tested whether 1 μM of chlorophylls will lead to further decrease in cell number. However, we could not observe the differences between 100 nM and 1 μM of chlorophyll, suggesting that saturation of chlorophylls' effect reached beyond 100 nM, at least. The number of cells was approximately 50% when compared to the control within 24 hour following chlorophyll treatment, possibly due to doubling time within 24 hours. When chlorophylls a and b were treated together, there was no significant difference in the absorbance data from the data obtained after independent treatments with chlorophyll a or b. These findings suggest that the inhibitory mechanism of chlorophyll a or b is not independent on each other.

Chlorophyll and its derivatives are very effective in binding with polycyclic aromatic hydrocarbons, heterocyclic amines, aflatoxin, and other hydrophobic molecules. These chlorophyll-carcinogen complexes are much harder to absorb by the body (Donaldson, 2004). Therefore, when green vegetables are consumed, it can be questionable whether the chlorophyll components can affect the cancer cells, which are remote from the gastrointestinal tract. However, in sera of volunteers, who had been taking the supplement, the presence of chlorophyllin, a chlorophyll derivatives has also been detected (Egner et al., 2000), indicating that chlorophyll can function in places other than the digestive tract (Egner et al., 2000). This *in-vitro* culture model bears the possibility of screening the effect of chlorophyll on the cancerous cells that are remote from the gastrointestinal system.

Previously, antioxidant intake and brain tumor risk has been reported, including ascorbic acid consumption (Blowers et al., 1997; Boeing et al., 1993; Bunin et al., 1994; Bunin et al., 1993; Giles et al., 1994; Hu et al., 1999; Kaplan et al., 1997; Lee et al., 1997). As for the glioblastoma in the brain, a strong association with oxidant stress has also been reported, assessing the influence of ascorbic acid and tocopherol (Schwartzbaum and Cornwell, 2000). Chlorophylls are known to possess anti-oxidative effect. Oxygen radical binds with chlorophylls or their derivatives and causes reduction in peroxy-lipid formation (Guzman et al., 2001; Hsu et al., 2005; Kamat et al., 2000; Kumar et al., 2001; Lee et al., 2003). Therefore, it is possible that effect of chlorophylls on GM growth could be mediated by anti-oxidative mechanism.

In conclusion, chlorophylls inhibited the growth of cultured U87 glioblastoma cells with alteration in PTEN phosphorylation, implying their role as a cytostatic agent.

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CONFLICT-OF-INTEREST STATEMENT

The authors declare no competing financial or academic interests.

REFERENCES

Balder, H. F., Vogel, J., Jansen, M. C., Weijenberg, M. P., van den Brandt, P. A., Westenbrink, S., van der Meer, R.,Goldbohm, R. A. (2006) Heme and chlorophyll intake and risk of colorectal cancer in the Netherlands cohort study. *Cancer Epidemiology, Biomarkers and Prevention* **15**, 717-725.

Blowers, L., Preston-Martin, S.,Mack, W. J. (1997) Dietary and other lifestyle factors of women with brain gliomas in Los Angeles County (California, USA). *Cancer Causes Control* **8**, 5-12.

Boeing, H., Schlehofer, B., Blettner, M.,Wahrendorf, J. (1993) Dietary carcinogens and the risk for glioma and meningioma in Germany. *International Journal of Cancer* **53**, 561-565.

Bunin, G. R., Kuijten, R. R., Boesel, C. P., Buckley, J. D.,Meadows, A. T. (1994) Maternal diet and risk of astrocytic glioma in children: a report from the Childrens Cancer Group (United States and Canada). *Cancer Causes Control* **5**, 177-187.

Bunin, G. R., Kuijten, R. R., Buckley, J. D., Rorke, L. B.,Meadows, A. T. (1993) Relation between maternal diet and subsequent primitive neuroectodermal brain tumors in

young children. *New England Journal of Medicine* **329**, 536-541.

Cheney, I. W., Johnson, D. E., Vaillancourt, M. T., Avanzini, J., Morimoto, A., Demers, G. W., Wills, K. N., Shabram, P. W., Bolen, J. B., Tavtigian, S. V.,Bookstein, R. (1998) Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated MMAC1/PTEN gene transfer. *Cancer Research* **58**, 2331-2334.

Chernomorsky, S., Segelman, A.,Poretz, R. D. (1999) Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth. *Teratogenesis, Carcinogenesis, and Mutagenesis* **19**, 313-322.

de Vogel, J., Jonker-Termont, D. S., van Lieshout, E. M., Katan, M. B.,van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis* **26**, 387-393.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J. P., Carraway, R., Reeves, S. A.,et al. (1995) Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron* **14**, 1075-1081.

Donaldson, M. S. (2004) Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutrition Journal* **3**, 19.

Egner, P. A., Stansbury, K. H., Snyder, E. P., Rogers, M. E., Hintz, P. A.,Kensler, T. W. (2000) Identification and characterization of chlorin e(4) ethyl ester in sera of individuals participating in the chlorophyllin chemoprevention trial. *Chemical Research in Toxicology* **13**, 900-906.

Egner, P. A., Wang, J. B., Zhu, Y. R., Zhang, B. C., Wu, Y., Zhang, Q. N., Qian, G. S., Kuang, S. Y., Gange, S. J., Jacobson, L. P., Helzlsouer, K. J., Bailey, G. S., Groopman, J. D.,Kensler, T. W. (2001) Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. *Proceedings of the National Academy of Sciences U S A* **98**, 14601-14606.

Furnari, F. B., Huang, H. J.,Cavenee, W. K. (1998) The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Research* **58**, 5002-5008.

Giles, G. G., McNeil, J. J., Donnan, G., Webley, C., Staples, M. P., Ireland, P. D., Hurley, S. F.,Salzberg, M. (1994) Dietary factors and the risk of glioma in adults: results of a case-control study in Melbourne, Australia. *International Journal of Cancer* **59**, 357-362.

Granick, S.,Beale, S. I. (1978) Hemes, chlorophylls, and related compounds: biosynthesis and metabolic regulation. *Advances in Enzymology and Related Areas of Molecular Biology* **46**, 33-203.

Guzman, S., Gato, A.,Calleja, J. M. (2001) Antiinflammatory, analgesic and free radical scavenging activities of the marine microalgae Chlorella stigmatophora and Phaeodactylum tricornutum. *Phytotherapy Research* **15**, 224-230.

Hawkins., A. J. S., Bayne., B. L., Mantoura., R. F. C.,Llewellyn., C. A. (1986) Chlorophyll degradation and absorption throughout the digestive system of the blue mussel Mytilus edulis L. *Journal of Experimental Marine Biology and Ecology* **96**, 213-223.

Hsu, C. Y., Yang, C. M., Chen, C. M., Chao, P. Y.,Hu, S. P. (2005) Effects of chlorophyll-related compounds on hydrogen peroxide induced DNA damage within human lymphocytes. *Journal of Agricultural and Food Chemistry* **53**, 2746-2750.

Hu, J., La Vecchia, C., Negri, E., Chatenoud, L., Bosetti, C., Jia, X., Liu, R., Huang, G., Bi, D.,Wang, C. (1999) Diet and brain cancer in adults: a case-control study in northeast China. *International Journal of Cancer* **81**, 20-23.

I., F. (1967) Absolute Configuration and the Structure of Chlorophyll. *Nature* **216**, 151-152.

Kamat, J. P., Boloor, K. K.,Devasagayam, T. P. (2000) Chlorophyllin as an effective antioxidant against membrane damage in vitro and ex vivo. *Biochimica et Biophysica Acta* **1487**, 113-127.

Kaplan, S., Novikov, I.,Modan, B. (1997) Nutritional factors in the etiology of brain tumors: potential role of nitrosamines, fat, and cholesterol. *American Journal of Epidemiology* **146**, 832-841.

Kumar, S. S., Devasagayam, T. P., Bhushan, B.,Verma, N. C. (2001) Scavenging of reactive oxygen species by chlorophyllin: an ESR study. *Free Radical Research* **35**, 563-574.

Lee, H. S., Choi, C. Y., Cho, C.,Song, Y. (2003) Attenuating effect of chlorella supplementation on oxidative stress and NFkappaB activation in peritoneal macrophages and liver of C57BL/6 mice fed on an atherogenic diet. *Bioscience, Biotechnology, and Biochemistry* **67**, 2083-2090.

Lee, M., Wrensch, M.,Miike, R. (1997) Dietary and tobacco risk factors for adult onset glioma in the San Francisco Bay Area (California, USA). *Cancer Causes Control* **8**, 13-24.

Li, D. M.,Sun, H. (1998) PTEN/MMAC1/TEP1 suppresses

the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proceedings of the National Academy of Sciences U S A* **95**, 15406-15411.

Lin, J., Chen, L., Lin, Z.,Zhao, M. (2007a) Inhibitory effect of triptolide on glioblastoma multiforme in vitro. *Journal of International Medical Research* **35**, 490-496.

Lin, J., Chen, L. Y., Lin, Z. X.,Zhao, M. L. (2007b) The effect of triptolide on apoptosis of glioblastoma multiforme (GBM) cells. *Journal of International Medical Research* **35**, 637-643.

Park, Y. J., Lee, W. Y., Hahn, B. S., Han, M. J., Yang, W. I.,Kim, B. S. (1989) Chlorophyll derivatives—a new photosensitizer for photodynamic therapy of cancer in mice. *Yonsei Medical Journal* **30**, 212-218.

Ruhle, W.,Paulsen, H. (2011) Preparation of native and recombinant light-harvesting chlorophyll-a/b complex. *Methods in Molecular Biology* **684**, 113-125.

Sarkar, D., Sharma, A.,Talukder, G. (1994) Chlorophyll and chlorophyllin as modifiers of genotoxic effects. *Mutation Research* **318**, 239-247.

Schwartzbaum, J. A.,Cornwell, D. G. (2000) Oxidant stress and glioblastoma multiforme risk: serum antioxidants, gamma-glutamyl transpeptidase, and ferritin. *Nutrition and Cancer* **38**, 40-49.