ORIGINAL ARTICLE

Gamma-tocotrienol Alters Protein Expression of HepG2 Cell Line

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ABSTRAK

Gamma-tokotrienol (GTT) telah menunjukkan aktiviti antitumor yang signifikan terhadap pelbagai sel tumor. Penemuan terdahulu menunjukkan bahawa GTT mempunyai kesan antiproliferasi terhadap sel kanser hepar (HepG2) pada nilai IC_{50} 170 µM. Di dalam kajian ini, kaedah elektroforesis gel dua dimensi (2DE) digunakan bagi mengetahui perubahan pada ekspresi protein di dalam sel HepG2 selepas rawatan GTT. Tujuan utamanya adalah untuk mengenalpasti mekanisme molekul yang mungkin terlibat dalam aktiviti antitumor GTT. Penumpuan diberikan kepada penghasilan profil protein 2DE sel HepG2 dengan dan tanpa rawatan GTT. Analisis awal terhadap profil 2DE, menunjukkan terdapat 18 titik protein diekspreskan secara berlainan di dalam sel dirawat GTT. Pemerhatian ini dipastikan dengan meluaskan lagi skop kajian kami kepada saiz sampel yang lebih besar. Dengan mengkaji kesan rawatan GTT kepada ekspresi protein di dalam sel HepG2, mekanisme asas yang terlibat pada sifat anti tumor secara diferensial GTT mungkin boleh dijelaskan.

Kata kunci: Proteomik, elektroforesis gel dua dimensi (2DE), Gamma-tokotrienol (GTT), sel HepG2

ABSTRACT

Gamma-tocotrienol (GTT) has been shown to exhibit significant antitumor activity in a variety of tumor cells. Previous findings have demonstrated that GTT had antiproliferative effects on a liver cancer cell line (HepG2) with an IC_{50} value of 170µM. In this study, two dimensional gel electrophoresis (2DE) was used to determine changes in protein expression in HepG2 cell line following treatment with GTT. The ultimate aim is to identify the possible molecular mechanisms involved in GTT antitumor activity. This study is focused on obtaining a 2DE protein profile for HepG2 cell line with and without GTT treatment. In the preliminary analysis of the resulting 2DE profiles, 18 protein spots were found to be differentially expressed in cells treated with GTT. This observation is confirmed by extending the analysis to a larger sample size. By studying the

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effects of GTT treatment on differential protein expression in HepG2 cells the underlying mechanisms involved in the antitumor activity of GTT may be elucidated.

Key words: Proteomics, two dimensional gel electrophoresis (2DE), Gamma-tocotrienol (GTT), HepG2 cells

INTRODUCTION

Vitamin E has been reported to have anticancer properties in both animal models and cells in culture (Nesaretnam et al. 1995). The vitamin E usually studied is α-tocopherol but recently more studies have focused on tocotrienol, isomers of vitamin E present in large quantities in palm oil. The tocotrienols are reported to be stronger antioxidants than α-tocopherol. In addition tocotrienols have properties which are different from α-tocopherol. In this laboratory, GTT have been shown to have antiproliferative effect against a variety of cell lines particularly liver cancer cells with an IC_{50} of 170 μM (Aida et al. 2007).

Among the tocotrienol group, delta-tocotrienol and gamma-tocotrienol are considered to have the strongest inhibitory effects on cancer cell growth (Sakai et al. 2004). Gamma-tocotrienol (GTT) has also been shown to exhibit antiproliferative properties on other tumor cells in culture, such as breast cancer cells (Nesaretnam et al. 1995), murine melanoma cells (He et al. 1997), human leukemia cells (Mo & Elson 1999) and malignant mammary epithelial cells (Shah & Sylvester 2005).

Several possible mechanisms of the antiproliferative effect of GTT have been suggested such as its involvement in inducing antioxidant effects (Noguchi et al. 2003; Calvisi et al. 2004), suppressive effects on HMG-CoA reductase activity (Parker et al. 1993; Mo & Elson 2004),

pro-apoptotic effects (Agarwal et al. 2004; Shun et al. 2004), regulating mitogenesis (Mo & Elson 1999) and anti-angiogenic potential (Miyazawa et al. 2004). In a recent and more elaborate study, it was shown that GTT inhibits the nuclear factor κB activation pathway, leading to down-regulation of various gene products and potentiation of apoptosis (Ahn et al. 2007). However, most of these mechanisms have only been studied on other types of tumor cells in particular breast cancer cell lines and specific pathways have not been fully characterized. To further understand the mechanisms involved in the anti-proliferative effects of GTT, it would be of interest to identify proteins whose expression are effected by exposure of HepG2 a liver cancer cell line to GTT treatment by using two-dimensional gel electrophoresis (2DE).

Proteomics has evolved into a robust and highly reliable method allowing researchers to profile cellular responses to various physiological and pathological conditions at the protein level. Currently it has been successfully employed in the area of cancer research, particularly in the search for new biomarkers (Cho 2007). Apart from that, it has also been utilized in the analysis of the response of tumor cells to an antitumor substance (Stockwin et al. 2007; Tong et al. 2008; Scheper et al. 2008). The majority of previous studies showed changes in the expression of proteins involved in the cellular processes such as apoptosis and proteins that are directly associated with

tumor development characteristic of the type of cancer (Yim et al. 2004; Mouat et al. 2005; Li et al. 2006; Tong et al. 2008).

In this study, 2DE was used to characterize changes in protein expression levels in HepG2 cell line following treatment with GTT. By identifying the specific alterations in the cellular protein profile in response to GTT treatment, the underlying mechanisms involved in the anti-proliferative activity of GTT may be elucidated.

MATERIALS AND METHODS

Cell culture and treatment

HepG2 and WRL68 cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were maintained in Eagle minimum essential medium (EMEM, Flowlab, Australia) containing 10% foetal calf serum (PAA, Austria), penicillin/ streptomycin (100 µg/ml) (Flowlab, Australia) at 37°C in an atmosphere containing 5% CO_2. Gamma-tocotrienol (GTT) was supplied by Malaysian Palm Oil Board (MPOB). Stock solution of GTT (0.5 M) was prepared in 100% ethanol and stored as small aliquots at -20ºC. Prior to use, GTT from stock solution was mixed with foetal calf serum and incubated overnight at 37ºC. Culture medium and 100% ethanol were then added to give a final concentration of 70 µM. When the cells reached confluency, they were trypsinized, centrifuged and counted using a haemocytometer. Cells were plated at a density of 2 x10^6 cells/100-mm culture plates for 24 hrs and incubated with 10 mL of 70 µM GTT enriched medium for 48 hours. As a negative control, cells were cultured in EMEM without GTT.

Sample Preparation for 2DE

At the end of the treatment period, cells were harvested by trypsinization and transferred into 15 ml Falcon centrifuge tubes. The cells were harvested by centrifugation at 800 rpm for 10 minutes. They were then washed thrice with cold PBS and the resulting pellet resuspended in 200 µl lysis buffer (8M urea, 2% CHAPS, 0.5% pH 3-10 IPG buffer, Amersham, USA) containing protease inhibitor mix (Amersham, USA). The cells were then incubated on ice for 30 minutes with intermittent vortexing at 10 minute intervals. After centrifugation at 13000 rpm for 30 minutes at 4ºC, the supernatant was transferred to sterile microcentrifuge tubes. Protein concentration was determined as described by Bradford (1976).

Two-dimensional Gel Electrophoresis

First dimension separation was carried out on immobilized pH gradient strips (24 cm, linear, pH 3-10, GE Healthcare) with Ettan IPGphor II Isoelectric Focusing System and standard strip holder (GE Healthcare). Isoelectric focusing (IEF) was performed under the following conditions: 500V for 1 hour, 1000V for 1 hour, 8000V for 3 hours and finally 8000V for 3 to 4 hours. Protein samples (60 µg) were loaded into sample cups at the anode end. Upon completion of IEF, strips were equilibrated in equilibration buffer (6M urea, 75 mM Tris-HCl, pH 8.8, 29.3% Glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) for 15 minutes, followed by the same buffer containing 25% iodoacetamide instead of DTT for another 15 minutes. The second dimension separation was carried out at 15ºC on 12.5% SDS slab gels using an ETTAN DALT II electrophoresis system (GE Healthcare), with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS-PAGE was run at constant power of 1 W/gel for 1 hour, then switched to 13 W/gel until the bromophenol blue marker reached the bottom of the gel. For every treatment group, triplicate runs were

made to ensure the accuracy of subsequent analysis.

Gel Staining and Image Analysis

Protein spots were visualized by silver staining as described in the PlusOne Silver Staining Kit (GE Healthcare). Images of stained 2DE-gels were acquired with a UMAX scanner, model UTA-2100 XL and stored as TIFF images. The 2DE maps were then analyzed using the 2D Image Master Platinum software Version 6.0 (GE Healthcare). The volume of each spot in a gel was normalized as a percentage of the total volume of all spots detected on the gel. Only those spots that were consistently changed (more than 2-fold) were selected as spots of interest in this study.

RESULTS

The proteome profile obtained from the analysis was highly reproducible between different runs and different sample sets. Representative gels are shown in Figure 1 and Figure 2. A total of ~500 individual protein spots were detected with silver staining. Following image analysis, a total of 83 spots were identified as being differentially expressed following GTT treatment. Out of the 83 spots, only 18 individual protein spots were differentially expressed in a consistent manner in every triplicate gel, and therefore, were selected as spots of interest in this study. These spots are shown in Figure 3.

DISCUSSION

In general, the 2DE gels were highly reproducible in terms of spot number, spot intensity and general protein profile. There were minimal intra and inter-sample variation among the 2DE gels. Consistency and reproducibility were achieved by standardized procedures of sample preparations of cells cultured, conditions for first and second dimensions of electrophoresis, gel staining and image acquisition. During sample preparation, a protease inhibitor cocktail was used. With the use of this inhibitor cocktail, resulting 2DE gels did not show any signs of protein degradation due to protease activity (as evidenced by the high resolution of high molecular weight proteins).
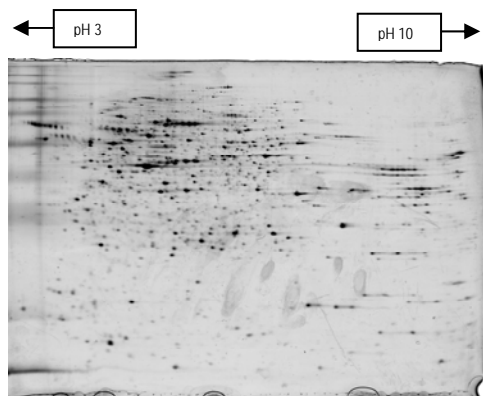

Figure 1: Representative 2D gel image of HepG2 cells without GTT treatment. Range of horizontal axis is from 3 to 10 pH units (left to right), while full range of vertical axis is from 10 to 250 kDa (bottom to top).
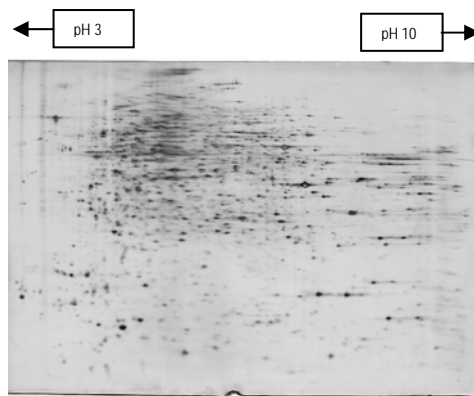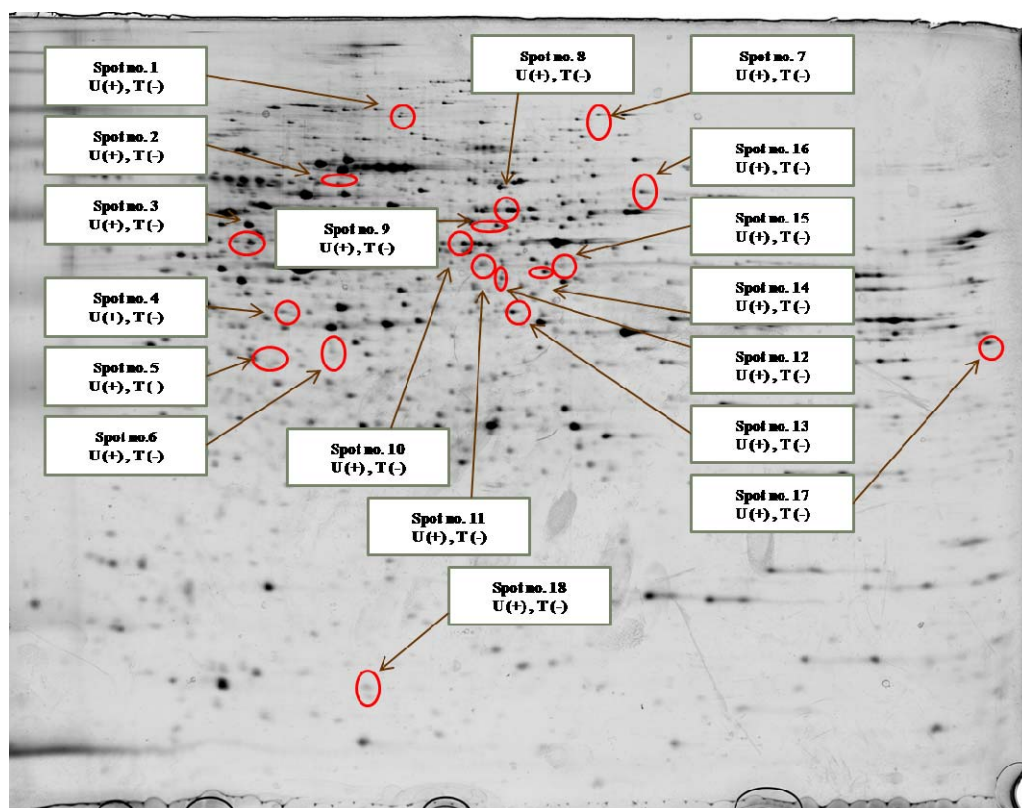

Figure 2: Representative 2D gel image of HepG2 cells with GTT treatment. Range of horizontal axis is from 3 to 10 pH units (left to right), while full range of vertical axis is from 10 to 250 kDa (bottom to top).

U, untreated HepG2 cell; T, treated HepG2 cell

(+), upregulated expression; (-), downregulated expression

Figure 3: Annotated 2DE map of HepG2. The boxed labels show the spot number and expression dynamics of the proteins of interest in this study.

Twenty four centimeter gels were used in order to obtain the best possible resolution for 2DE. A sample loading of 80µg protein per gel resulted in 2DE profiles with saturated protein spots which may lead to inaccuracies during quantitative analysis. A representative gel of an 80µg protein load is shown in Figure 4. To resolve this problem, a sample loading of 60µg protein per gel was used. This was found to be ideal for resolving the highest number of spots without sacrificing gel resolution. Image analysis revealed that there were no saturated spots present at this protein loading therefore ensuring that image analysis would remain quan-

titative. Thus at this point, we have established a robust and reliable protocol for the 2DE analysis of the proteome of HepG2 cell lines. We then moved on and performed differential proteomics analysis of treated and untreated HepG2 cell lines.

A total of 18 differentially expressed protein spots were identified following treatment of HepG2 cells with GTT. Among the protein spots, 7 of them (spot no 1, 2, 3, 4, 5, 6 and 18) are acidic (pI < 5) proteins. Of these seven, protein spot numbered 1 has the largest molecular weight (>90 kDa), while protein spot numbered 18 is the smallest sized pro-

tein with molecular weight around 10 kDa. The rest (spot no 2, 3, 4, 5 and 6) are medium-sized proteins with a molecular weight ranging from 30 to 50 kDa. A group of 8 protein spots (spot no 8, 9, 10, 11, 12, 13, 14 and 15) are found accumulated in the middle of the 2D-PAGE gel of both group of HepG2 cells (with and without GTT treatment). All of these proteins have a molecular weight ranging from 35 to 60 kDa. The pI of these proteins could vary from slightly acidic, neutral, to slightly basic. This cannot be confirmed because all the proteins were too close between each other. Another two (spots no 7 and 16) are assumed as slightly basic proteins since they were both well-separated on the slightly basic region of the 2D-PAGE gel. These spots were large proteins with the molecular weight around 60 to 90 kDa. The last protein from the 18 differentially expressed proteins were highly basic proteins with spot numbered 17 as average-sized protein (around 30 kDa). These observations showed that the changes in protein expression occur over a wide pH and molecular weight range and were not localized to any specific region of the gel. This was expected as treatment with GTT will most likely affect a wide variety of proteins.
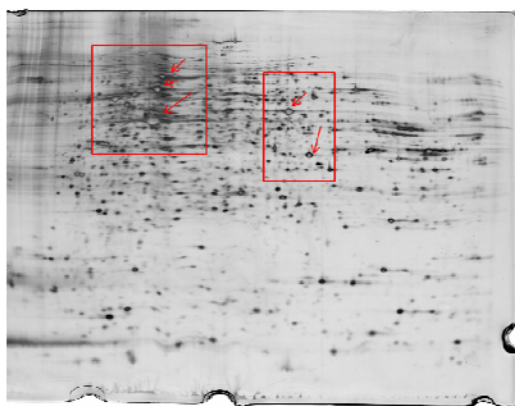
Interestingly, all of these 18 protein spots were observed only in HepG2 cells without GTT treatment and not in the HepG2 cells with the GTT treatment. In other words, these proteins were greatly decreased to the extent that they were no longer detectable in the 2D protein profile of HepG2 cells with GTT treatment. Whether these proteins are directly related to the antiproliferative activity of GTT remains unclear as we have yet to identify these proteins.

Our results demonstrated that GTT treatment caused specific and significant changes in the 2DE profile of the HepG2 cell line. Proteins generally function in groups that are involved in many biological pathways simultaneously. Thus, any interventions will ultimately affect the regulation and/or modulation of not only one but many proteins simultaneously. This has been continuously demonstrated in various studies of the intracellular mechanisms of various drugs. One example is the study conducted by Ahn et al. (2007), demonstrated that GTT suppressed NF-κB activation pathway and NF-κB-regulated gene products. As in our study, the unknown proteins that are involved might be associated with the anti-tumor activity of GTT in HepG2 cells. Thus, further identification of these proteins by mass spectrometry may lead to a greater understanding of the mechanisms involved in the anti-proliferative effects of GTT.

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Figure 4: Representative 2D gel image of HepG2 cells with 80 µg protein loading. Saturated protein spots are shown by the arrows.

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