NMR metabolomics of drug response to antineoplastic polyherbal formulations studied in human hepatocellular carcinoma cells

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Introduction: Cancer is a leading cause of death worldwide and poses a huge health challenge. Among the treatment modalities for cancer, chemotherapy plays an important role. Many of the chemotherapy drugs are plant based and there is continuing interest in evaluating the anticancer properties of medicinal plants (1). There is also a growing appreciation of the use of polyherbal formulations since these are considered to have low toxicity and are also multitargeting (2). The present study has used NMR metabolomics to profile the drug response of human Hep-G2 cancer cells, considered resistant to conventional chemotherapy. The treatment in this study has been administered by four polyherbal formulations, whose antineoplastic activities have also been assessed using apoptosis (programmed cell death) detection assay (3). The cytotoxicity of these formulations on Hep-G2 cells have been previously reported (4).

Materials and Methods: Four polyherbal formulations labeled KG, VK, GTK and MK were selected for study. The formulations were made up of medicinal plants such as *Commiphora mukul, Emblica officinalis, Terminalia chebula, Terminalia bellerica, Piper nigrum, Piper longum and Raphanus sativus*.

Antineoplastic activity: 4 x 10^4 Hep-G2 cells were plated into each well of 4 sets of 12-well plate and treated for 48 hours with the conventional chemotherapeutic drug paclitaxel (positive control, 10 µg/ml) and 50, 75 & 100 µg/ml of formulations. One set of cells was left untreated. Apoptosis detection assay was carried out using BD Annexin V (A)- Fluorescein isothiocyanate (FITC) Propidium Iodide (PI) apoptosis detection kit and evaluated by flow cytometer (BD LSR II FACS). Cumulative cell death (early and late apoptosis) resulting from treatment was assessed.

NMR: 5x10^6 Hep-G2 cells were treated with formulations (100 µg/ml) for 48h. The cells were then extracted using chloroform-methanol and water dual phase extraction method (5), lyophilised and redissolved in 600 µl of 100mM phosphate buffer (pH 7.0) prepared in 90% H_2O-10% deuterated trimethylsilyl propionate (TSP). Eight replicate samples were prepared per treatment and were pooled for spectroscopic analysis. Water suppressed 1D proton spectra of the extracts of untreated and treated Hep-G2 cells were acquired on 700 MHz NMR spectrometer (Varian, USA) using the following acquisition parameters: spectral width - 12 ppm, relaxation delay - 4s, no. of scans - 64, data points - 32 K. Peaks were assigned using BML-NMR library (6). Principal Component Analysis (PCA) of the spectral data was carried out using SPSS v.20 software.

Results and Discussion: Of the three concentrations (50, 75 and 100 µg/ml) evaluated, 100 µg/ml showed maximum antineoplastic activity. Amongst the four formulations, VK registered maximum cell death. The data presented below is, therefore, for VK at 100 µg/ml. Figure 1 shows the flow cytometry data for untreated Hep-G2 cells, and cells treated with paclitaxel and VK. The cumulative cell death for VK is 98.8% and that of paclitaxel is 86.6%. Figure 2 shows proton NMR spectra of untreated Hep-G2 cell extracts and those after treatment with paclitaxel and the four formulations. Untreated Hep-G2 cells showed prominent resonances in the region between 3.5-3.8 ppm (predominantly glycerol) and also from metabolites such as isoleucine, lactate, pyruvate, citrate, dimethylamine and choline (Fig.2a). The treatment by paclitaxel, VK and KG induced drastic reduction of the glycerol peaks (Figs. 2b, 2c, 2e). There are also other interesting spectral changes such as-appearance of L-alanine with paclitaxel treatment, and β-alanine with VK, GTK and KG treatment. The decrease in lactate with treatment could reflect the positive response of Hep-G2 cells to the drugs suggesting the hindered glycolysis in Hep-G2 cells. The PCA plot of the NMR data (Fig. 3) shows that the spectral profiles of all drug responses are different from untreated cells. It is interesting to see the spectral similarity in drug response to paclitaxel and KG..

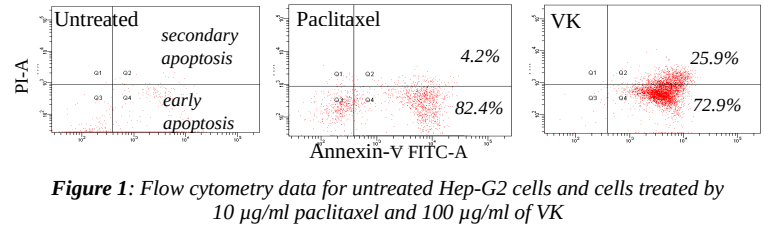

Figure 1*: Flow cytometry data for untreated Hep-G2 cells and cells treated by 10 µg/ml paclitaxel and 100 µg/ml of VK*

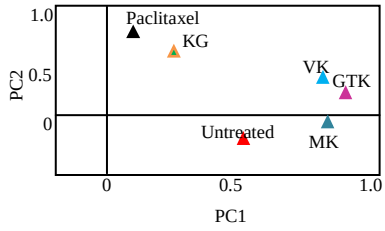

Figure 3: *PCA plot of the NMR spectral bins of untreated cells, and cells treated with paclitaxel, KG, MK, VK and GTK.*

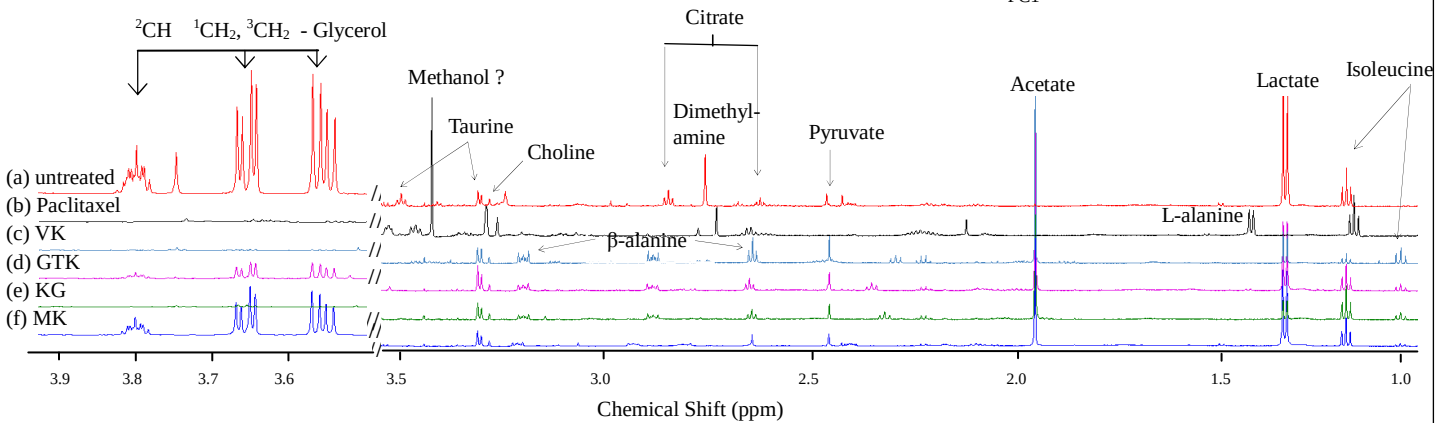

Figure 2: *Proton NMR metabolomic profiling of Hep-G2 cells (a) untreated and treated with (b) paclitaxel (c) KG, (d) GTK, (e) MK (f) VK*

Conclusion: NMR profiling of drug induced changes have been demonstrated for the first time for polyherbal formulations, all of which showed antineoplastic activity. Drastic reduction in peaks were observed in 3.5-3.8ppm. This study also provides a first time evidence of polyherbal formulations causing apoptosis in cancer cells.

References: (**1**) Cragg GM *et al. Chem Rev*, 109:3012-4370, 2009 (**2**) Lee KW *et al. Nat Rev Cancer*, 11:211-218, 2011 (**3**) Lodi A *et al. Plos One*, 6, 2011 (**4**) Jayasundar R *et al. ISMRM*, 20:1059, 2012. (**5**) Sellick CA *et al. Metabolomics*, 6:427-438, 2010 (**6**) Tiziani S *et al. Plos One*, 4, 2009.