Pattern of mobile lipid accumulation in HeLa cells during paclitaxel-induced cell death using diffusion-weighted spectroscopy.

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Intro: Paclitaxel is a cytotoxic drug which acts by destabilising microtubules resulting in mitotic arrest and subsequent apoptosis. Although a positive relationship between apoptotic cells and ^{1}H NMR observable lipid resonances (MLR) has been detected[1-4]using NMR spectroscopy the nature and degree of change in lipid saturation is still unclear. Understanding the pattern of observed changes would help establish MLR as potential biomarker of drug response. In this study, changes in MLR and their saturation following exposure of HeLa cells to the anti-mitotic drug paclitaxel were investigated using diffusion-weighted (DW) HR-MAS spectroscopy.

Methods: The HeLa cervical cell line (Cancer Research UK Cell Services, UK) was cultured under standard conditions. Cells were exposed to 1μM paclitaxel (99%ethanol) (Sigma, UK) 24 h post-inoculation for 8, 16, 24 and 48 hours (n=3). All spectra were acquired using a Bruker Avance 11.74 T spectrometer (Bruker BioSpin, Germany), ^{1}H frequency of 500 MHz, equipped with a 4mm triple resonance ^{1}H/^{31}P/^{13}C HR-MAS probe with a gradient aligned along the magic angle axis. Diffusion-weighted spectra were acquired using a stimulated echo sequence, with bipolar gradients[5] (ledpbgs, Bruker, Germany), repetition time (TR), 4.76 s; echo time (TE), 10.21 ms; time between diffusion gradients (Δ), 100 ms; diffusion gradient length (δ), 10 ms; gradient amplitude 95% of 55 G/cm (52.25 G/cm); spectral width, 10,000 Hz; data size, 32 K; 128 transients. For all measurements, samples were spun at 3 kHz and temperature maintained at 277 K. The intensity of the 3.0 ppm peak from lysine-containing cytoplasmic polypeptides was very stable within diffusion-weighted spectra of exposed and control cells and therefore was used as an internal standard. Figures 1 and 2 show relative peak intensities of MLR normalised to lysine peak at 3.0 ppm. TOPRO-3 and nile red co-staining (Sigma, UK), flow cytometry and confocal microscopy were employed to measure cells in order to visualise cell cycle phase distribution, apoptotic features, and accumulation of cytoplasmic lipid droplets (droplets size not determined). Trypane Blue (TB) staining determined cells viability and plasma membrane integrity (apoptotic cells).

Results: Figure 1 shows the accumulation of MLR in HeLa cells undergoing paclitaxel-induced programmed cell death. No significant changes in peak intensities at 1.3 and 0.9 ppm (Figure 2) were seen before 24h, after which a significant increases (Table 2) were observed together with an increase in the 5.3 ppm peak (Figure 3, Table 3). At 48h a further significant increase in the intensities of MLR peaks was recorded, including the polyunsaturated peak at 2.8ppm (Figures: 2, 3, Table 1). At 24h more than 90% of the cells were detached of which 65% of cells were growth arrested at G2/M phase of the cell cycle and 35 % displayed apoptotic nuclear morphology. More than 70% (±3) of cells had an intact plasma membrane as indicated by TB staining. The number of lipid droplets per cell increased significantly ($p<0.0001$) to 2.84±0.4 in exposed cells (0.58±0.25 in controls) (Figure 4). At this stage more than 85% (±3) cells exposed to paclitaxel were apoptotic, 8% (±4) were arrested in G2/M phase of the cell cycle but not apoptotic. 32 % (±4) of cells had an intact plasma membrane. The number of lipid droplets per cell increased significantly ($p<0.001$) from to 12.11±3.2 from (2.345±0.4) (Figure 4).

Discussion: Application of diffusion-weighted spectroscopy simplified analysis of NMR spectra, by removing signals originating from low molecular weight metabolites. Stability of the 3.0 ppm peak from lysine-containing metabolites provided a useful internal standard. The observed pattern of MLR changes is consistent with two-step MLR accumulation (at 0.9 and 1.3 ppm) reported by Brisdelli *et al*[3], reflecting effector and degradative phases of apoptosis. Our data also shows changes in unsaturated lipids during paclitaxel-induced apoptosis. This change (reflected by the 5.3 ppm peak) occurs at 24h and seems to be greater than the corresponding changes in 1.3 and 0.9 ppm peak intensities. A significant change in the intensity of the polyunsaturated peak at 2.8 ppm in cells undergoing programmed cell death is in agreement with literature data[1] proving that polyunsaturated lipid formation is characteristic of apoptotic cell death. Although studies with nile red using confocal microscopy confirmed a significant increase in cytoplasmic lipid droplet formation a precise determination of their size distributions is being performed to correlate observed MLR changes with the lipid droplet content of the cells.

Conclusions: Paclitaxel-induced apoptosis is associated with an increase of MLR including rise in unsaturated lipids at 24h that occurs as a result of lipid droplet formation. The pattern of changes in unsaturated lipids observed by ^{1}H NMR may serve as a non-invasive time-course biomarker of apoptotic cell death.

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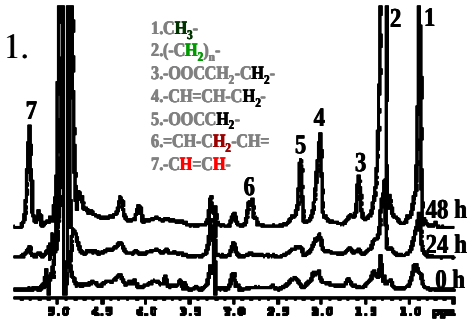

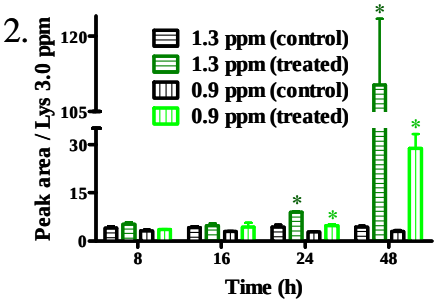

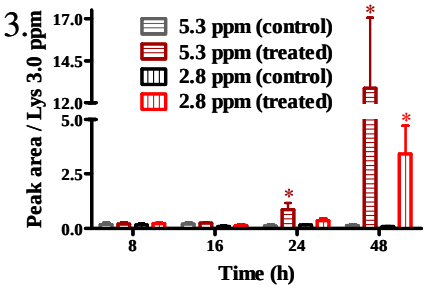

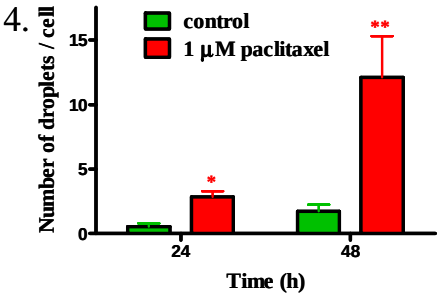

Table 1. Increase in MLR (multiples above baseline)

	0.9 ppm	**1.3** ppm	**2.8** ppm	**5.3** ppm
24h	1.6**	2.1***	2.2	6.8*
48h	9.5***	25.5***	34.1**	85.1**

increases significant at: *p<0.05, **p<0.01, ***p<0.001