Comparison of NMR lipid profiles in mitotic arrest and apoptosis as indicators of drug resistance.

D. Zietkowski[1], E. Nagy[2], M. A. Mobberley[3], G. S. Payne[1], T. A. Ryder[3], and N. M. deSouza[1]

[1]CRUK and EPSRC Cancer Imaging Centre, The Institute of Cancer Research, Sutton, Surrey, United Kingdom, [2]Section of Molecular Carcinogenesis, The Institute of Cancer Research, Sutton, Surrey, United Kingdom, [3]Department of Cellular Pathology, Imperial College Healthcare NHS Trust, London, United Kingdom

Background: Although an increase in mobile lipids in apoptosis has been previously reported (1,2) and is an indicator of treatment response, the changes in their saturation for identifying resistance to treatment has not been explored. Cell culture systems offer the opportunity to establish the timing of the alteration in the lipid fractions in various stages of the cell cycle, growth arrest and apoptosis in sensitive and resistant lines. This may be measured using diffusion-weighted NMR spectroscopy with which it is possible to quantify the mobile fraction of lipids and observe changes in their saturation. The purpose of this study, therefore, was to measure and characterise changes in NMR visible saturated and unsaturated lipids and their temporal pattern in paclitaxel sensitive (HeLa) vs. resistant C33A, (Me180) cervical cancer cell lines following exposure to paclitaxel in order to explore their utility as a biomarker of drug resistance.

Methods: Cervical cell lines: HeLa (CR UK Cell Services, UK), C33A and Me180 (ATCC, USA) were 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). Spectra of cell pellets were acquired using a Bruker Avance 11.74 T spectrometer (Bruker BioSpin, Germany), 1H 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 (DW) spectra were acquired at 277 K using a stimulated echo sequence, with bipolar gradients (3) (Bruker, Germany), repetition time (TR), 4.76 s; echo time (TE), 20.4 ms; time between diffusion gradients (Δ), 100 ms; diffusion gradient length (δ), 10 ms; gradient amplitude 520 mT/m; spectral width, 10,000 Hz; data size, 32 K; 128 transients. Cellular morphology was assessed using transmission electron microscopy (TEM) using uranyl acetate followed by lead citrate staining.TOPRO-3 and Nile red co-staining confocal microscopy were employed to measure cells in order to visualise cell cycle phase distribution, apoptotic features, and accumulation of cytoplasmic lipid. Trypan Blue (TB) staining determined cells viability, size and plasma membrane integrity (apoptotic cells). Western blots were used to assess activity and expression of phospholipase A2 ($cPLA_2$, P-$cPLA_2$) and fatty acid synthase (FASN).

Results: No significant changes in the DW lipid profiles were observed before 24 hours. At this time point the morphology of cells assessed using TEM microscopy indicated the presence of mitotic arrest in all cell lines (>60% of cells) with apoptosis in Hela samples. At 48 hours HeLa cells progressed relentlessly to apoptosis while C33A and Me180 cells showed progression beyond mitotic arrest to normal morphology or multinucleation (Fig.1) indicating resistance. Examples of DW spectra of HeLa cells at both 24 and 48h are shown in Fig.2. Figures 3 shows increases in saturated lipids at 1.3 ppm (CH_2-C**H**$_2$-CH_2) , Fig.4 shows changes in unsaturated lipids at 5.3 ppm (-C**H**=C**H**-) at these timepoints in sensitive HeLa and resistant C33A cells (similar pattern was seen for Me180). Table 1 summarises overall fold increases in MLR peaks above controls in all cell lines. Also the1.3/0.9 ppm ratio changed dramatically at 48 h up to 2.7 in Me180 and C33A (Table 1). Analysis using TEM revealed a significant increase (*) in an average droplet size (Fig.5a) in sensitive HeLa cells, whereas in resistant cell lines a significant decrease (**) in average droplet size at 48h was seen. The percentage of cells displaying droplets on TEM increased in all lines (Fig.5b). Confocal microscopy showed an increase the number of droplets per cell (Fig. 5c). The expression levels of FASN, c-PLA_2 and phosphorylated (activated) P-$cPLA_2$ were unchanged (slightly decreased) in HeLa cells at 24h after exposure followed by a decrease at 48h when P-$cPLA_2$ was no longer detectable. However in C33A and Me180 lines, $cPLA_2$ was stably expressed throughout the exposure to paclitaxel and its activated form P-$cPLA_2$, which was not detectable in control samples, was detected in both lines after 24 and 48 h exposure to paclitaxel.

Discussion: Mitotic arrest was seen with both TEM and flow cytometry (not shown) in all cell lines at 24h consistent with paclitaxel entering the cell and interfering with the stability of microtubules. C33A and Me 180 cells activated mechanisms of resistance which allowed them to progress beyond mitosis and either return to normal morphology or accumulate multiple nuclei. Lipid peak intensities distinguished between sensitive and resistant cells. The highest fold increase seen in the polyunsaturated lipid diallyl peak at 2.8 ppm in all cell lines was independent of the presence of apoptotic cells in the sample, and may be more indicative of mitotic growth arrest. Apoptosis is mainly associated with an increase in (poly-) unsaturation of fatty acid chains of triglycerides and increased lipid visibility reflected by massive increase in methylene over methyl ratio. Lipid signals are likely to have originated from lipid droplets, which were larger than in controls in sensitive HeLa cells at both 24 and 48 h, but did not increase in size in resistant lines. Paclitaxel resistant cells accumulated less unsaturated lipids which were not necessarily triglycerides. Increased expression of FASN and $cPLA_2$ does not seem to be responsible for the observed lipid increases in paclitaxel induced apoptosis. The lack of change in the expression levels of FASN in resistant lines and $cPLA_2$ activation (only detected in resistant cells) exposed to paclitaxel indicates continued activity of synthesis and degradation of lipids despite resistance. Changes in the methylene over methyl ratio are significantly lower in resistant cells.

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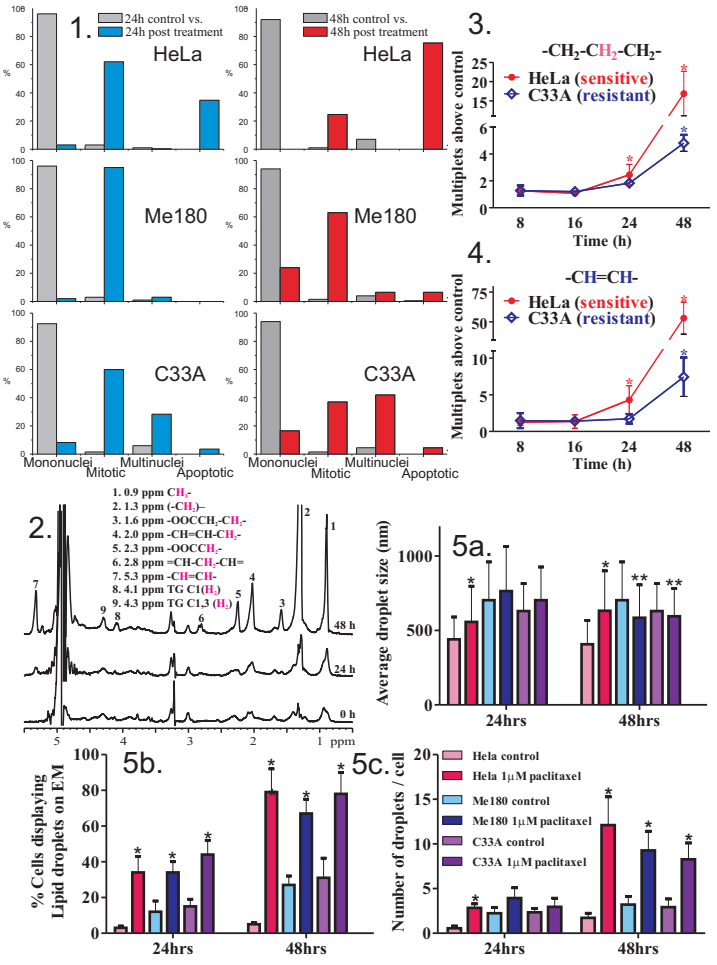

Table 1

HeLa	0.9 ppm	1.3 ppm	1.6 ppm	2.0 ppm	2.3 ppm	2.8 ppm	4.1 ppm	4.3 ppm	5.3 ppm	1.3/0.9 ppm
1μM paclitaxel at 24h	1.81x	2.38 x	8.56x	1.57x	1.35x	2.96x	1.29x	1.4x	4.01x	1.48 » 1.95
1μM paclitaxel at 48h	**6.46x**	**16.72x**	40.84x	5.15x	4,53x	**17.99x**	6.39x	2.43x	**68.03x**	**1.46 » 3.77**
Me 180										
1μM paclitaxel at 24h	1.69x	2.05x	2.5x	1.3x	1.3x	3.49x	1.08x	1.16x	3.69x	1.29 » 1.65
1μM paclitaxel at 48h	**2.8x**	**4.02x**	4.94x	2.7x	2.31x	**5.22x**	3.19x	1.63x	**5.83x**	**1.99 » 2.74**
C33A										
1μM paclitaxel at 24h	1.48x	1.83x	1.8x	1.3x	1.41x	2.62x	1.75x	1.4x	1.74x	1.72 » 2.12
1μM paclitaxel at 48h	**2.5x**	**4.75x**	5.8x	1.95x	1.37x	**2.12x**	1.33x	1.36x	**8,63x**	**1.47 » 2.77**