[1]H MAS NMR spectroscopy of metabolites and lipids during cell growth arrest induced by cisplatin in cultured rat glioma BT4C cells.

L. Mirbahai[1], M. Wilson[2], C. S. Shaw[1], C. McConville[2], R. D. Malcomson[3], R. A. Kauppinen[4], and A. C. Peet[2]

[1]School of Sport and Exercise Sciences, University of Birmingham, Birmingham, West midlands, United Kingdom, [2]School of Cancer Sciences, University of Birmingham, Birmingham, United Kingdom, [3]Department of Histopathology, Birmingham Childern's Hospital NHS Foundation Trust, Birmingham, United Kingdom, [4]Dartmouth Medical School, Hanover NH, United States

Introduction

Magnetic resonance spectroscopy (MRS) can be used clinically to complement magnetic resonance imaging (MRI)[1] by non-invasively identifying important molecular biomarkers.In particular; there is interest in identifying markers of early response to therapy[2,3,4] allowing timely changes in treatment strategy. In this study alterations of lipid and metabolites in rat glioma cells *in vitro* were investigated via [1]H MAS NMR spectroscopy after exposure to cisplatin for various time intervals. Transmission electron microscopy and Nile red stained images were achieved to determine the source of visible lipids. In addition to Trypan blue (TB) and H& E staining, DAPI in combination with Nile red was used; to detect the viability status of the treated cells. Propidium iodide stained, cisplatin treated BT4C cells were investigated by flow cytometry to determine the stage of growth cycle.

Methods

Rat glioma BT4C cells were cultured in supplemented D-MEM F:12 medium and exposed to 50μM cisplatin for 12, 24, 48 or 72h. [1]H MAS NMR spectroscopy was performed on the harvested untreated and treated cells via a Varian 600 MHz spectrometer with a probe temperature of 0.1°C using a pulse and acquire sequence with 256 scans, a repetition time of 3.3s and spinning rate of 2500Hz. Spectra were phased and referenced to the creatine peak at 3.01ppm and then an extended version of the automated TARQUIN[5] analysis method was used to quantify metabolites and lipids. Nile red staining was applied to identify the source of NMR visible lipids. The lipid droplet area within each cell and the diameter of the cytoplasmic lipid bodies were measured using ImageJ software. DAPI and Nile Red co-staining was performed to assess the viability status and the method of cell death (apoptosis or necrosis) in lipid droplet containing cells. Images were taken using a Nikon E600 fluorescent microscope coupled with a SPOT RT KE colour 3 shot CCD camera. Haematoxylin and eosin (H&E) and Trypan blue staining were carried out on cells to provide further evidence on the percentage of viable and dead cells after treatment at different time intervals. In addition Transmission electron microscopy images were taken to investigate the ultrastructure and viability status of the cells containing lipid droplets. To determine the stage of cell cycle and to investigate the possibility of cell growth arrest in cisplatin exposed cells at different time points, the cells were stained with propidium iodide (PI) and analysed by flow cytometry, and the cell cycle distribution of the cells was explored by multicycle software.

Results

Increase in unsaturated and saturated fatty acid moieties were detected at frequencies of 0.89, 1.3, 1.58, 2.02, 2.2, 2.8, and 5.4ppm, with longer exposure to cisplatin (Fig. A). The increase in NMR visible lipids was detected along with an increase in lipid droplet diameter and lipid droplet area in each cell (Fig. B). An increase in Glycerophosphocholine (GPC) and a decrease in phosphocholine (PC) was detected; resulting in a decline in the ratio of PC/GPC with longer cisplatin incubation. An increase in choline, lactate and alanine and a decrease in taurine levels were also identified at longer time intervals (Fig. A). Trypan Blue and H&E staining reveal a small percentage of dead cells at 12, 24, 48h, however the number of dead cells increased significantly after 72h. In addition DAPI staining confirmed these results and illustrated the presence of lipid droplets in the cells that appeared morphologically viable (Fig. C). TEM images also confirmed the presence of lipid droplets in cells which are morphologically viable (Fig D). Flow cytometry results indicated that with longer exposure to cisplatin, the cells accumulate in the G1 stage of the cell cycle, representing growth arrest in this stage.

Discussion and conclusion

An increase in NMR visible lipids associated with intra-cytoplasmic lipid droplets was detected at early stages of cisplatin treatment (12, 24, and 48 h) when a high percentage of cells appeared morphologically viable. Changes in key metabolites were also detected during this stage of treatment. Flow cytometry confirmed that the changes were associated with cell cycle arrest. A decline in the ratio of PC/GPC before the onset of apoptosis has been associated previously with cell growth arrest[1]. This study raises the possibility of using increases in saturated and unsaturated lipids and metabolite alterations as early biomarkers of cisplatin treatment efficacy; prior to the appearance of any morphological characteristics of apoptosis.

References: 1. Hakumaki, J.M., et al., (1999) Nat Med, 5, 1323-7 **2**. Robinson, S.P., et al., (1997) Br J Radiol 70 Spec No, S60-9 **3**. Ronen, S.M., et al., (1999) Br J Cancer, 80, 1035-41 **4**. Milkevitch, M., et al., (2005) Biochim Biophys Acta, 1734, 7-12 3. **5**. Reynolds, G., et al., (2006). Magn Reson Med, 56, 1211- 9

Figures: A. Spectra of untreated and cisplatin treated cells. **B**. Mean percentage of the area covered by lipid droplets in each cell (the error bars are SEM, *$p<0.05$ and **$p<0.001$; paired t-test). **C**. Nile red and DAPI stained image of 24h cisplatin exposed BT4C cells. The arrow head is pointing to a necrotic cell and the arrow is showing an apoptotic cell. **D**.TEM image of 24h cisplatin exposed BT4C cell. The arrows are pointing to lipid droplets and the arrow head is showing a swollen mitochondria. (Funded by MRC, UK).