MAPPING PHOSPHORYLATION OF PEPTIDE BIOMARKERS CORRESPONDING WITH GLIOBLASTOMA TUMOURS USING DDA AND A NOVEL MRT MASS SPECTROMETER

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INTRODUCTION

Glioblastoma is an aggressive form of brain tumor with little therapeutic options available, and correspondingly the 5-year survival rate remains <10% of those diagnosed, whilst the median life expectancy is 15 months [1]. Phosphorylation is a key post-translational modification (PTM) that is involved in numerous processes, such as activation of key enzymes/kinases for example. Hence, phosphorylation sites are key targets for the regulation and development of glioma and may offer insight for new targeted therapies to perturb specific cellular pathways [2].

Profiling of phosphorylated residues using mass spectrometry can be problematic due to the labile nature of the phosphoryl group. For example, during collision induced dissociation the group can be lost (H_3PO_4 or HPO_3 + H_2O) resulting in a neutral loss of –98Da. When combined with data independent acquisition (DIA) approaches and complex spectra, it can make identification of phosphorylated residues difficult. However, utilising data dependent acquisition (DDA) mode allows relation of the precursor *m/z* and that resulting from a neutral loss to that of the quadrupole set mass, facilitating identification of phosphorylated peptides [3].

Here, we describe an LC-MS workflow using the Xevo™ MRT Mass Spectrometer; a novel benchtop high resolution, high mass accuracy platform for accurately and definitively characterizing these post-translational modifications whilst also providing corresponding quantitative data (Figure 1).

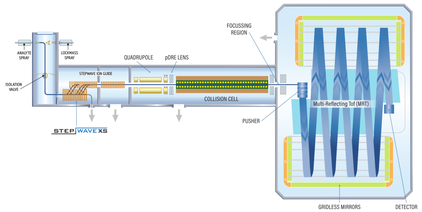

Figure 1. Schematic of the Xevo MRT Mass Spectrometer showing the newly implemented collision cell alongside the gridless Tof demonstrating multiple reflections of the ion beam leading to 100,000 FWHM mass resolution.

METHODS

Adult primary glioblastoma cell lines (four) were treated with arginine deprivation therapy using arginine degrading enzyme ADI-PEG20. Treated and untreated cell lines were lysed in the presence of protease and phosphatase inhibitors (Figure 2). Samples were then reduced, alkylated and tryptically digested prior to enrichment using Fe(iii)-NTA spin columns. The LC-MS platform consisted of an ACQUITY™ Premier chromatography system that was specially coated to minimise non-specific adsorption thereby increasing phosphopeptide recovery coupled to a Xevo MRT MS. Enriched phosphopeptides were separated using a 30 min gradient. MS data were collected using the DDA mode, where the top 15 most intense ions per survey scan were selected for respective MS/MS.

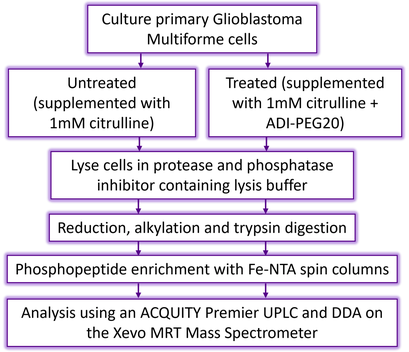

Figure 2. Experimental workflow followed from growing of cells through to MS analysis following in vitro treatment of cells with arginine deprivation [4].

Conditions

LC	
System	ACQUITY Premier UPLC™
Column	ACQUITY Premier Peptide CSH™ columnn 2.1 x 100mm (p/n 186009488)
Column temperature	45°C
Solvents	A; H2O + 0.1% FA, B; MeCN + 0.1% FA
Flow	150µL/min
Gradient	1 to 35% B over 30 mins, followed by a wash at 85% B
MS	
System	Xevo MRT
Acquisition mode	Data dependent acquisition
MS Survey range	50-2000 m/z
MS Survey scan rate	10Hz
MS/MS ions	Top 15 ions
MS/MS scan rate	4Hz
Peak detection	Charge state detection; 2+, 3+, 4+
Dynamic peak exclusion	Acquire and exclude for 30 seconds, 500mDa window
Collision energy	Charge state recognition collision energy ramp

RESULTS

Phosphorylated peptides were selectively enriched and subject to analysis by data dependent acquisition on the Xevo MRT MS. Data generated from the workflow in Figure 2 were processed using ProteinLynx Global SERVER™ (PLGS).

Due to the relatively low proportional amount of phosphorylated peptides compared to unmodified sequences, enrichment prior to analysis was necessary in order to characterise these low abundant molecules.

- Fe(iii)-NTA spin columns were utilised to selectively bind phosphopeptides, allowing unmodified peptides to flow through.
- Initial experiments indicated an abundance of phosphorylated peptides identified through neutral loss of 98Da within the enriched sample and so a full study analysis was completed
- Data dependent acquisition successfully switched on the top 15 most intense ions and provided excellent MS/MS spectra for site localisation of phosphorylated residues (Figure 3)
- Fast scanning, combined with low interscan-delay and high resolution of the Xevo MRT MS facilitated identification of over 320 proteins (Figure 5)

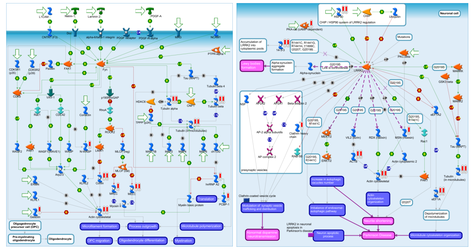

Figure 4. Pathway analysis of identified proteins in a single cell line of untreated vs treated. Identified proteins are mapped onto the "Regulation of cytoskeleton proteins in oligodendrocyte differentiation and myelination" (left) and "LRRK2 in neurons in Parkinsons".

- Pathway mapping of identified proteins using MetaCore™ revealed, as expected, pathways associated with neurological disorders and regulation of cytoskeleton proteins (Figure 4)
- Process networks indicated to be involved were also focussed around translation, cytoskeleton regulation and protein folding
- Translational networks showed differences between the untreated and treated cells with the metabolic therapy

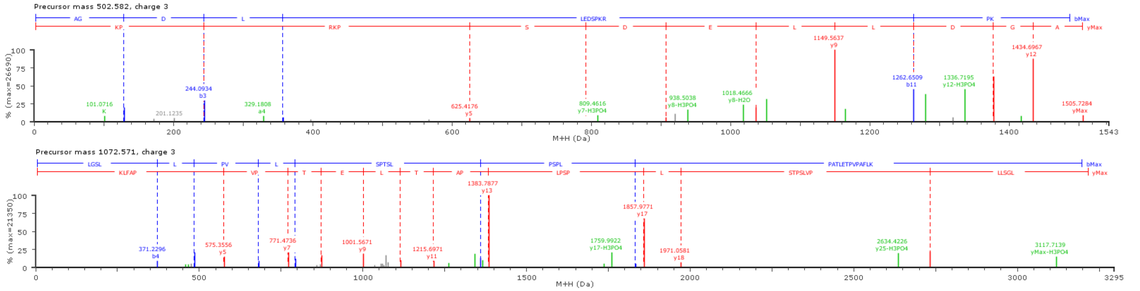

Figure 3. Fragmentation spectra of matched primary ions plus neutral losses of peptide with sequences AGDLLEDpSPKRPK (top) LGSLLPVLSPTpSLPpSPLPATLETPVPAFLK (bottom). Loss of phosphoryl group observed as a neutral loss of 98Da are shown in green.

DISCUSSION

Identification of phosphopeptides is of particular difficulty due to their low abundance in biological samples and labile nature of the modification during CID leading to inability to sufficiently localise sites of phosphorylation. Nevertheless, employing enrichment strategies coupled to DDA on a benchtop Xevo MRT MS successfully profiled the phosphoproteome of human glioblastoma cell lines before and after treatment with a metabolic therapy.

Mapping the effect of metabolic therapies on protein activation through network analysis provides insights and shapes future strategies for drug discoveries. In this case, the metabolic therapy applied to the glioblastoma cell lines demonstrated marked differences in protein identifications compared to the untreated cell lines. Key areas of interest were identified as including activation pathways involving kinases as expected, but also neurological pathways and regulation of cytoskeleton proteins.

Further work to understand the downstream implications of these effects is currently underway to elucidate the mechanism of action of the metabolic therapy.

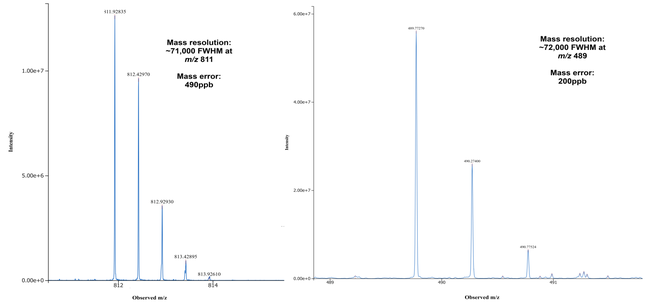

Figure 5. MS1 spectra of the doubly charged peptide with sequence KQSLGELIGTLNAAK (m/z 811.92835) and AALLKASPK (m/z 489.77270). Mass resolution was >71,000 FWHM and mass error was <500ppb.

CONCLUSION

- **Phosphopeptide enrichment from human glioblastoma cell lines was successfully implemented using Fe(iii)-NTA spin columns**
- **ACQUITY Premier demonstrated excellent non-retention of phosphopeptides**
- **Data dependent acquisition successfully profiled and localised sites of phosphorylation**
- **Xevo MRT MS provided unparallelled mass resolution of >70,000 FWHM at fast scan speeds of 20Hz and allowed for highly confident, unambiguous identification of both precursor and fragment ions**

References

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