

Introduction

A pathological feature of Alzheimer's disease (AD) is the accumulation in brain neurons of abnormally hyperphosphorylated tau. This results in its dissociation from micro-tubules and polymerisation into tangles of paired helical filaments (PHF) as shown in the light microscopy image below (Figure 1).

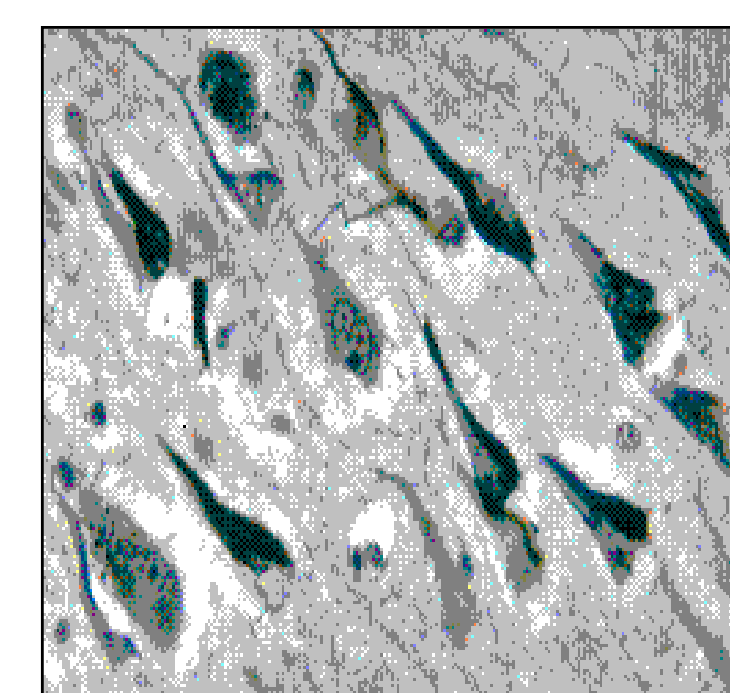


Figure 1: Light microscopy of AD brain

Neurofibrillary tangles - Such aggregates are responsible for neuronal cell death, a central feature of AD. Currently there are two tau-related biomarkers that have been evaluated in human CSF, namely total tau (T-tau) and phospho-tau (P-tau).

More recently CSF P-tau assessments have focused on different phospho-epitopes but these are limited to a few sites within the protein.

The determination of the phosphorylation sites on PHF tau has been undertaken using mass spectrometry (MS) and many new sites have been discovered¹. Antibodies to detect many of these sites do not currently exist and it is both a challenging and lengthy process to produce reagents with the appropriate selectivity and specificity. Recent developments in MS now enable the screening of tau phosphorylation sites independent of disease status, species of origin, or location within the protein. We present results to demonstrate the application of MS to the analysis of tau phosphorylation in clinical and pre-clinical samples. It is envisaged that such methods will provide the basis for the most comprehensive phospho-site specific array for tau available within the AD research community.

Phospho-Tau Discovery Studies

Tau was enriched by biochemical techniques using protocols we have previously established and demonstrated for both human brain samples as well as material for transgenic mice. Gel electrophoresis is used as the final step in this process. Tau related bands are then excised from the gel and an enzymatic digest is performed to produce a mixture of peptides and phosphopeptides. A panel of enzymes was used to extend the sequence coverage of the protein and, in some instances, provide an overlapping set of phospho-peptides. Each digested sample was then analysed using a combination of LC-MS/MS (Ultimate LC system, Dionex, coupled to Q-ToF *micro*, Waters) and dedicated precursor m/z 79 scans (Qtrap 4000, Applied Biosystems). All MS/MS spectra relating to phosphopeptides were visually verified to confirm the phosphorylation site indicated.

The LC/MS/MS analysis of PHF-tau revealed many sites of phosphorylation on serine and threonine residues including 12 novel sites. Additionally phosphorylation of Tyr394 was also shown to be present² (Figure 2). Identification of these new sites has led to further studies to identify the specific kinases involved in the phosphorylation of PHF tau. We have characterised the phosphorylation sites of recombinant tau phosphorylated by known selected kinases and also a rat brain cell lysate, which contains a pool of cellular kinases. This has allowed us to closely emulate PHF tau phosphorylation and discover the key kinases involved.

Importantly, these experiments indicate that casein kinase 1 delta (CK-1δ) can phosphorylate tau at additional sites to those modified by the more established tau kinases such as GSK3β and CDK5. We also demonstrate that c-Abl is the kinase responsible for in vivo phosphorylation of Tyr 394 in PHF-tau.

Figure 2 Phosphorylation sites on PHF-tau from AD brain

mAEPRQEFEV MEDHAGTYGL GDRKDDGGYT MHQDQEGDTP AGLKESPTQT	50
PTEDGSEEPG SETSDAKSPFAEDVTFAPLV DEGAFGKQAA AQPHTIPEFG	100
TTTAEAGIGD TFSDEEAAG HVTQARmvsK skdgtgsddk kagdadgkttk	150
iatprgaapp gqkQANATR IPAKTPPAPK TPPSGEPPK SDRSGYSSP	200
GSPGTPGSRs RTPSLPTPT REPKKVAVVR TPPKSPSAK SRLQTAPVPM	250
PDLKNVSKIKI GSTENLKHQP GGGKVIINK KLDLSNVSK cgskdniKHV	300
PGGGIVQIVY KFDLSKVTs KCGSLGNIHH KPGGGQVEVK SERLDFKDRV	350
QSKIGSLDNI THVPGGNKK IETHKLTFRE NAKAKTDHGA EIYKSPVVS	400
GDTSPRHLSN VSSGIDMV DSPQLMLAD EVSLLAKOG L	441

Development of Phospho-Tau SRM assays

Selective reaction monitoring (SRM) MS has been used for many years as a diagnostic and forensic tool to accurately quantify the levels of toxins and drug metabolites. SRM is the scan type with the highest duty cycle and is used for monitoring one or more specific ion transition(s) at high sensitivity. Here, Q1 is set on the specific parent m/z (Q1 is not scanning), the collision energy is set to produce the optimal diagnostic charged fragment of that parent ion, and Q3 is set to the specific m/z of that fragment (Figure 3A). Only ions with this exact transition will be detected.

The feasibility of using SRM as the basis of site directed tau phosphorylation assays has been demonstrated and is illustrated below (Figure 3B). The area under the SRM LC peak is used to quantitate the amount of the analyte present. In a typical quantitation experiment, a standard concentration curve is generated using synthetic versions of the phosphopeptides of interest. An 11 point calibration curve (0.25 – 1000 fmol) is produced of light synthetic phosphopeptides with each point in the curve spiked with 100 fmol heavy phosphopeptide. When the unknown sample is then run under identical conditions (i.e. 100 fmol heavy peptide spiked into the sample), the concentration for the phosphopeptide in the unknown sample can be determined using the standard concentration curve.

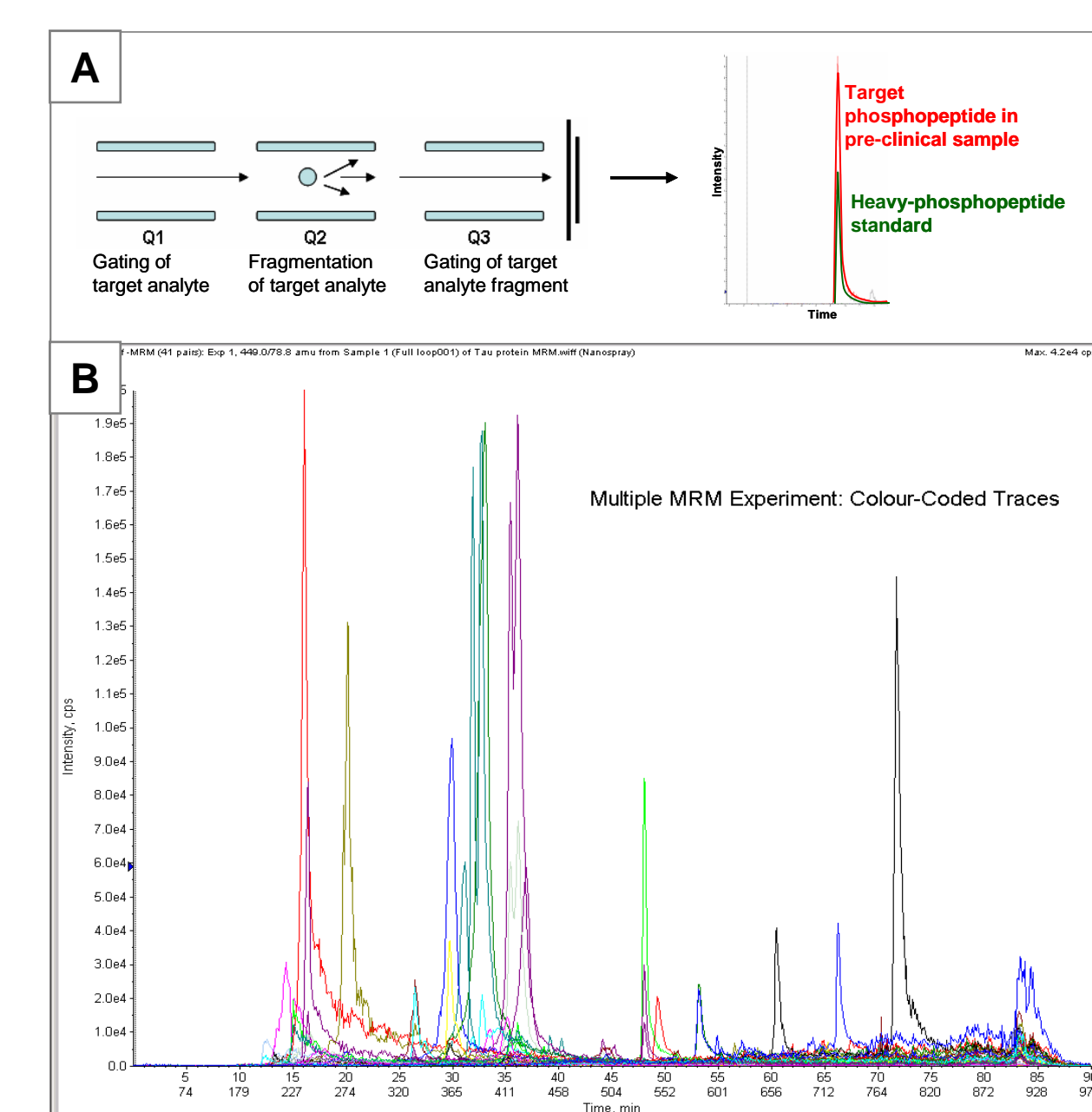


Figure 3 SRM assays for tau phosphorylation

- Panel A shows a schematic of an SRM experiment measuring light and heavy transitions.
- Panel B shows the total ion chromatogram (TIC) generated for 41 SRM transitions designed to selectively monitor 15 phosphorylation sites within tau protein.
- In this example PHF-tau from post mortem human brain was purified and enzymatically digested using trypsin.
- Assay criteria can be optimised for particular sites and the inclusion of synthetic phosphopeptides enables the absolute concentrations of each individual phosphopeptide to be established.

Phospho-Tau SRM Assay v1

Method

The Phospho-Tau SRM assay measures the presence of phosphate at key sites (threonine 170, serine 188, threonine 220 & serine 385) within tau isolated from the brains of mice used in pre-clinical studies of AD. Each phosphopeptide was monitored by three or six SRM transitions (42 SRM transitions in total, representing light and heavy versions of five tau phosphopeptides, Figure 4), using optimised SLens values and collision energy settings.

The method quantifies the phosphorylation in pre-clinical material using a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific). Prior to SRM analysis phosphopeptides and pre-clinical samples were resolved by RP-chromatography (XBridge column, Waters) over a 9 minute gradient 0-30% ACN (buffer A; 0.1% FA, buffer B; ACN, 0.1% FA). SRMs were visualised through Pinpoint (Thermo Scientific) and all peak matching visually verified. For generation of calibration curves (0.25-1000 fmol light peptide spiked with 100 fmol heavy peptide) transitions were summed to give a total intensity for all transitions for each phosphopeptide.

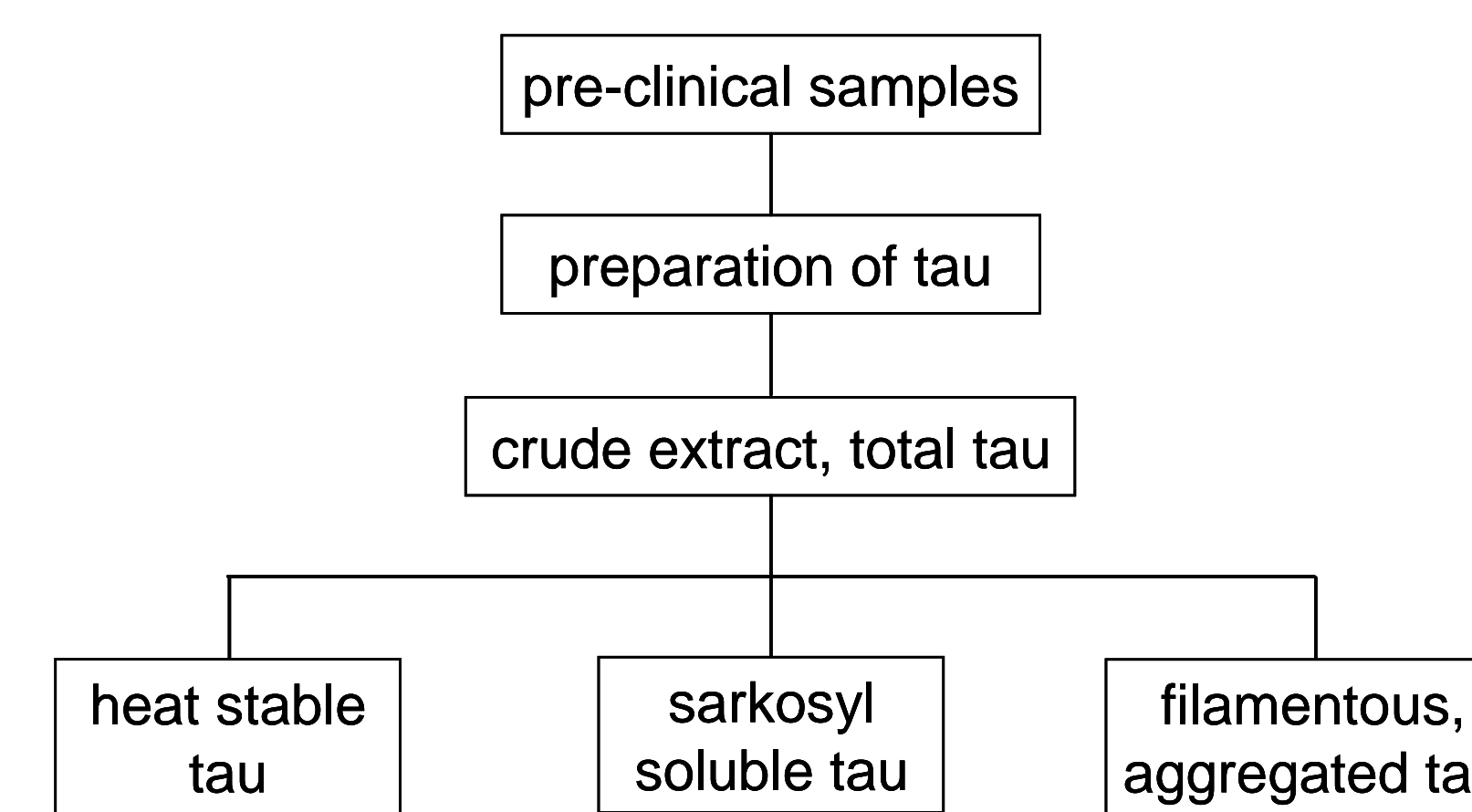
Figure 4 Phosphopeptide standards

pS or pT = phosphorylated amino acid; P = ¹³C x 5, ¹⁵N proline

	Sequence	Phospho-residue	Residue number (mouse)	Residue number (human, 2N4R)
Peptide 1	TTPSPKpTPPGSGEPPK	threonine	170	181
Peptide 2	TTPSPKpTPPGSGEPPK			
Peptide 3	TTPSPKpTPPGSGEPPKSGER	threonine	170	181
Peptide 4	TTPSPKpTPPGSGEPPKSGER			
Peptide 5	SGYSpSPGSPGTPGSR	serine	188	199
Peptide 6	SGYSpSPGSPGTPGSR			
Peptide 7	TDHGAEIVYKpSPVVSVDTSR	serine	385	396
Peptide 8	TDHGAEIVYKpSPVVSVDTSR			
Peptide 9	VAVVRpTPPKSPSASK	threonine	220	231
Peptide 10	VAVVRpTPPKSPSASK			

Mouse cortex tissue from either wild type C57BL/6 or P301L transgenic mice were homogenised at 100 mg/mL and centrifuged. Tau protein was partitioned into four fractions to provide an analysis of distinct populations of phosphorylated tau proteins (Figure 5). Following gel electrophoresis, coomassie stained bands of interest were excised, digested with trypsin and analysed using the Phospho-Tau SRM assay v1 method. For each specified tau population, the endogenous level of each tau phosphopeptide was quantified against its calibration curve. Prior to LC-SRM analysis each tau population was spiked with 100 fmol of each of the heavy phosphopeptide standards.

Figure 5 Preparation of distinct populations of phosphorylated tau



Assay Performance criteria

- 42 SRM transitions monitor five phosphopeptides covering four key phospho-tau sites, namely threonine 170, serine 188, threonine 220 & serine 385 (according to mouse residue numbering)
- The working assay range is 500 attomoles – 1 picomoles
- The LOD and LOQ values are phosphopeptide dependent but typically in the range of 25-50 attomoles (LOD) and 500 attomoles (LOQ)
- Replicate measurements (n=10) for phosphopeptide 10 demonstrate a CoV of 3.4%

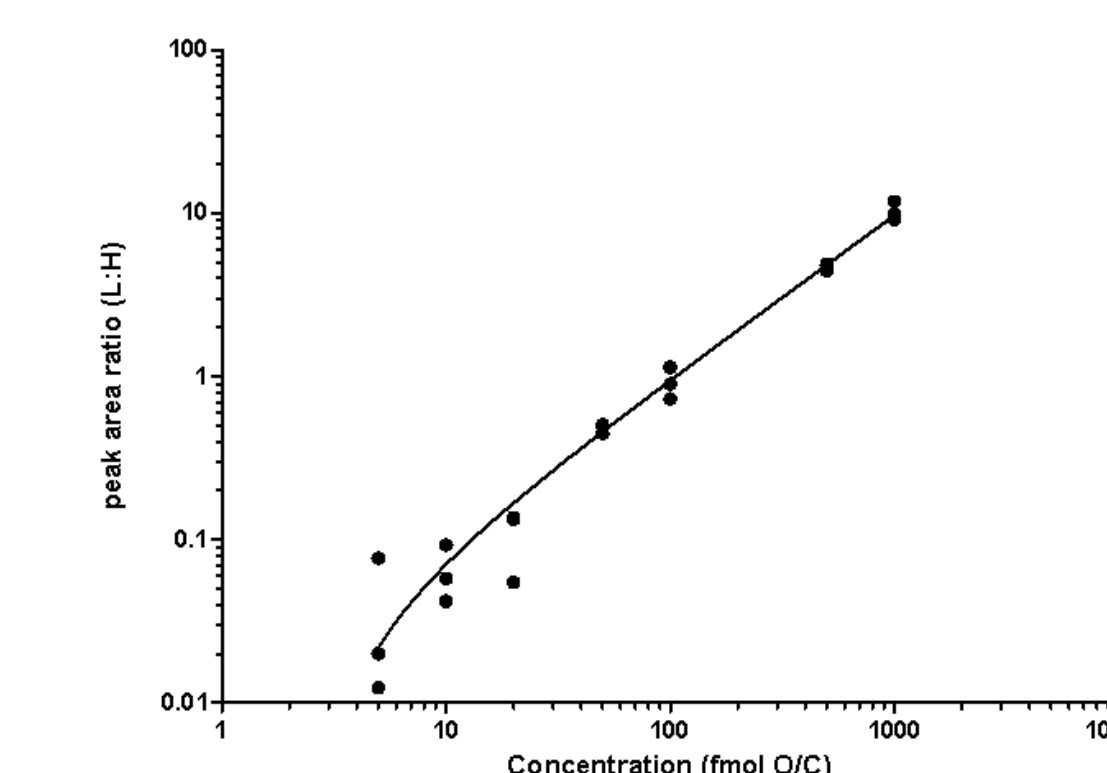


Figure 6 Calibration curve for phosphopeptide SGYSpSPGSGTPGSR.

Calibration curve (0.25 – 1000 fmol) of the light peptide, spiked at each concentration with 100fmol of the heavy peptide.

Assay implementation in a pre-clinical mouse model of AD

The Phospho-Tau SRM assay v1 was applied to the analysis of phosphorylated tau populations extracted from cortex of wild type, C57BL/6 and AD transgenic, P301L mice (n=1) as described above. An increase (23%) in levels of pSer 188 is observed in filamentous, aggregated tau in P301L mice relative to C57BL/6 mice (Figure 7).

Figure 7 Relative concentration of pSer 188 in C57BL/6, wild type and P301L, AD transgenic mouse models.

Tau fraction	Relative concentration of pSer 188	
	C57BL/6 (pg/ug total protein)	P301L (pg/ug total protein)
Crude extract, total tau	18.0	24.9
Heat stable tau	60.9	158.7
Sarkosyl soluble tau	17.9	25.5
Filamentous, aggregated tau	2.2	51.1

Conclusions

- MS workflows have been established and are available to screen and quantify phosphorylation sites in tau
- PhosphoTau SRM Assay v1 enables the simultaneous screening of four phosphorylation sites in tau isolated from pre-clinical materials.
- Further reiterations of the method will include additional sites of phosphorylation and provide improved sensitivity to extend the utility of these assays into clinical studies.

References

- Hanger *et al* (2007) Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *J Biol Chem.* 282(32):23645-54.
- Derkinderen *et al* (2005) Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase. *J Neurosci.* 25(28):6584-93.