Label Free LC-MS Based Proteomics for Integrated Preclinical Pharmaceutical Toxicology
Experience from the FP6 InnoMed PredTox Consortium

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Overview:

A label free LC-MS analysis the liver of rats treated with a compound inducing significant liver damage (liver cell necrosis, bile duct inflammation, bile duct proliferation), bile duct necrosis and hypertrophy of the hepatocytes). The method demonstrates excellent analytical reproducibility (CV = 10.9 %), and good proteome coverage (595 proteins identified at 6% false identification rate) with relatively modest instrument time required per sample. The dataset was sufficient to perform an informative pathway analysis.

The InnoMed PredTox Consortium:

Toxicity and safety issues remain a significant problem for drug development efforts by pharmaceutical and biotechnology companies. Specifically, current early biomarkers of toxicity are insufficient and this is demonstrated by the high failure rate of candidate therapeutics due to toxicity problems in both preclinical and clinical stages of development. PredTox (www.innomed-predtox.com) is a collaborative project partly funded by the EU involving a consortium of 15 industrial (13 large pharma, 1 technology provider and 1 SME) and 3 academic partners (see figure 1). The stated aim of this consortium is to assess the value of combining data generated from 'omics technologies (proteomics, transcriptomics, metabonomics) with the results from more conventional toxicology methods to facilitate more informed decision making in preclinical safety evaluation, and Initiate and support the development of scientists within the novel field of Systems Toxicology.

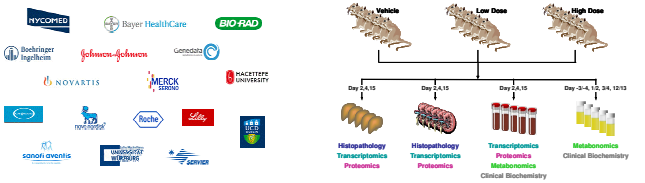


Figure 1 – The InnoMed PredTox Consortium. The consortium is a partnership between pharmaceutical companies, small-medium enterprises, and academic institutions in Europe. A combined 'omics approach is used in animal models of pharmaceutical toxicity in an effort to improve pre-clinical safety evaluation.

Methods:

- Liver protein extracts were prepared from 5 rats treated daily for 14 days with high dose LCB 3343 and 5 rats treated with vehicle control.
- 100 µg of the liver protein extracts were reduced, alkylated, aceone precipitated, re-solubilised using an acid labile surfactant, trypsin digested and re-suspended in 10.1 % formic acid, 3 % (v/v) acetonitrile.
- The trypsin digested protein samples were analysed using an 1200 Series nanoLC connected online to a 6520 QTOF mass spectrometer via an orthogonal electrospray Chipcube interface (Agilent Techonologies). 3 ug of digested protein was chromatographed with a 90 minute gradient from 3 % (v/v) acetonitrile, 0.1 % (v/v) formic acid to 40 % (v/v) acetonitrile, 0.1 % formic acid using a HPLC-Chip equipped with a 75µm x 150mm, 5µm C-18 300SB-Zorbax analytical column and a 160 nl Zorbax 300SB-C18 5µm enrichment column. The mass spectrometer was operated using two duty cycles for this analysis, one to maximise the information content of the MS1 scans. (1 MS scan for 333 milliseconds, and 2 MS/MS scans in 333 milliseconds), and one to maximise the number of peptide identifications (1 MS scan for 125 milliseconds, and 8 MS/MS scans for 333 milliseconds).
- Raw data files were converted to the open mzXML format using the Trapper converter (Institute for Systems Biology) and imported into Progenesis LC-MS (Nonlinear Dynamics) for feature detection, alignment, quantification and statistical analysis.
- Additional LC-MS/MS runs using inclusion lists were carried out to target peptides which were statistically significant but were not identified in the original analyses.
- MS/MS spectra were searched using Mascot (Matrix Science) against version 3.53 of the International Protein Index Rat database with a reversed decoy database (for false identification rate calculation) and common contaminant proteins appended.
- Pathway analysis was generated using Ingenuity Pathway Analysis (Ingenuity Systems)

Results:

- 19,749 peptide features detected above the selected intensity threshold of 1000 (40,447 features in total)

- 595 proteins identified (false identification rate of 6 % by decoy database search)

- 90 proteins determined to differentially modulated between treatment and vehicle control groups (fold change > 1.5 and ANOVA p-value < 0.05)

- Coefficient of variation for 6 replicate injections of a pooled sample is 10.9 %

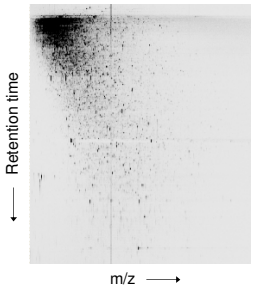
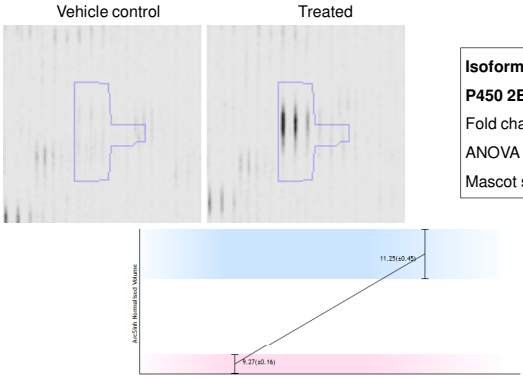


Figure 2 – 2-dimensional display of the peptide LC-MS analysis

Differentially modulated peptide



Isoform 1 of Cytochrome P450 2B2 (IPI00193234)
Fold change = 7.5
ANOVA p-value = 1.58E-06
Mascot score = 57.55

Figure 3 – Example of a differentially modulated peptide which maps to isoform 1 of Cytochrome P450 2B2.

Technical variation

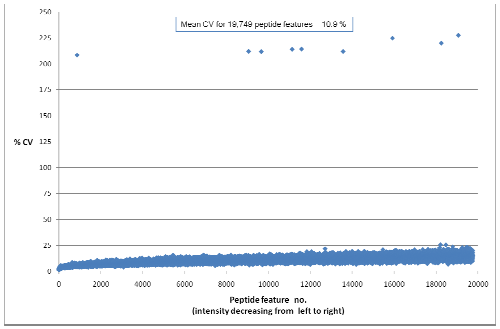


Figure 4 – Plot displaying the % coefficient of variation across 6 technical replicate injections of a pooled liver sample. Virtually all of the peptides are below a CV of 25 % and the mean is 10.9%

Results:

Ingenuity Pathway Analysis

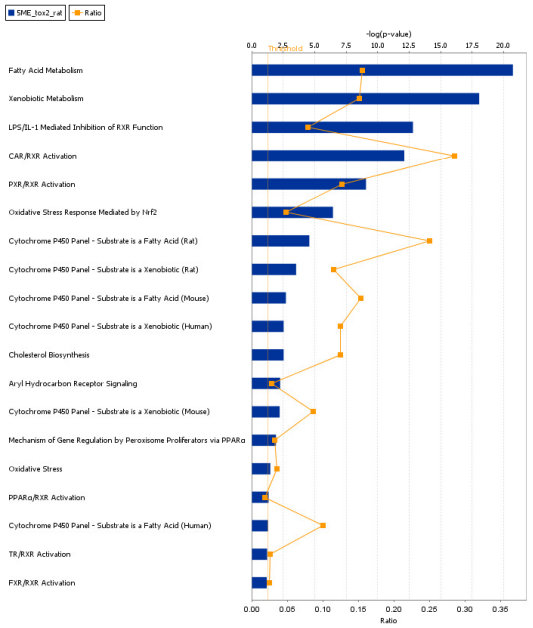


Figure 5 – Most significant regulated pathway lists as determined by Ingenuity Pathway Analysis

Results Summary and Discussion:

- Technical variance for this method is low (mean CV = 10.9 %)
- ~20,000 peptide features are detected and quantified
- 595 proteins identified at 6 % false identification rate (by decoy database search)
- The majority of peptides that are quantified are not assigned sequence annotations
- Further work is required to increase the number of peptide and protein annotations (additional inclusion list optimisation and potentially pre-fractionation of the pooled sample)
- Pathway analysis is possible and useful at this level of proteome coverage to generate toxicologically relevant hypotheses

Acknowledgements:

We would like to thank all other members of the PredTox Consortium. Funding is acknowledged under the EU FP6 Integrated Project, InnoMed. Funding is also acknowledged from Science Foundation Ireland and Agilent Technologies. The UCD Conway Institute and the Proteome Research Centre is funded by the Programme for Research in Third Level Institutions (PRTLI), as administered by the Higher Education Authority (HEA) of Ireland.