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) is presented. The method demonstrates excellent analytical reproducibility (CV = 10.9 %), and good proteome coverage (809 proteins identified at 3.1 % false identification rate) with relatively modest instrument time required per sample. The dataset was sufficient to perform an informative pathway analysis and a meaningful comparison with a previous gene expression analysis of the same samples.

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) was 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.

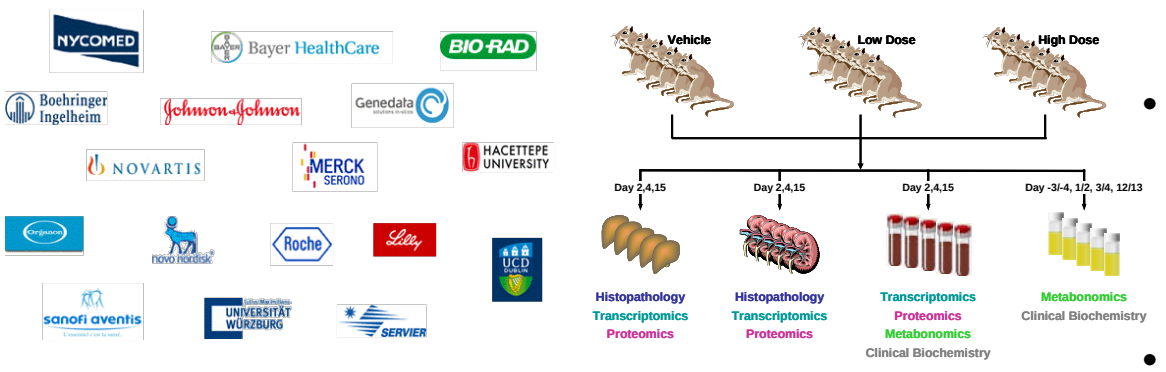

Figure 1 – The InnoMed PredTox Consortium. The consortium is a partnership between pharmaceutical companies, small-medium enterprises, and academic institutions in Europe. A combined 'omics approach is used in animal models of pharmaceutical toxicity in an effort to improve pre-clinical safety evaluation.

Methods

- Liver protein extracts were prepared from 5 rats treated daily for 14 days with high dose liver toxicant (study FP005ME) and 5 rats treated with vehicle control.
- 100 µg of the liver protein extracts were reduced, alkylated, acetone 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 using a reversed phase nanoflow separation with on-line microfluidic-based nanoelectrospray Q-ToF MS and MS/MS (Agilent Technologies). 3 µg 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 spectra. (1 MS spectrum for 333 milliseconds, and 2 MS/MS spectra in 333 milliseconds), and one to maximise the number of peptide identifications (1 MS spectrum for 125 milliseconds, and 8 MS/MS spectra 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)

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Results and Discussion

- 19,749 peptide features detected above the selected intensity threshold of 1000 (40,447 features in total – see Figure 2 for representative LC-MS map)
- 809 proteins identified via 2,797 peptides (false identification rate of 3.1 % by decoy database search, 717 proteins with 2 or more peptides)
- 90 proteins determined to be differentially modulated between treatment and vehicle control groups (fold change > 1.5 and ANOVA p-value < 0.05) – see Figure 3 for an example peptide and Figure 5 for volcano plot of the entire data set.
- Coefficient of variation for 6 replicate injections of a pooled sample is 10.9 % (see Figure 4)

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

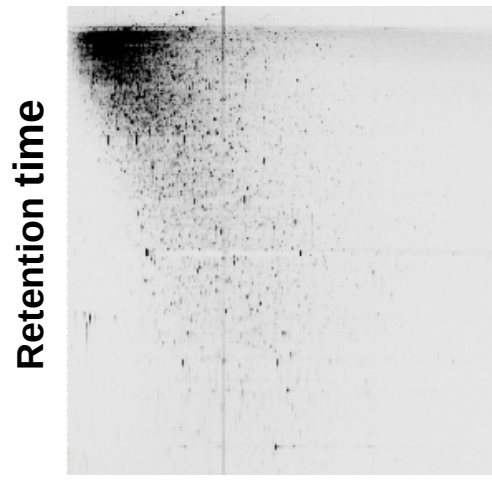
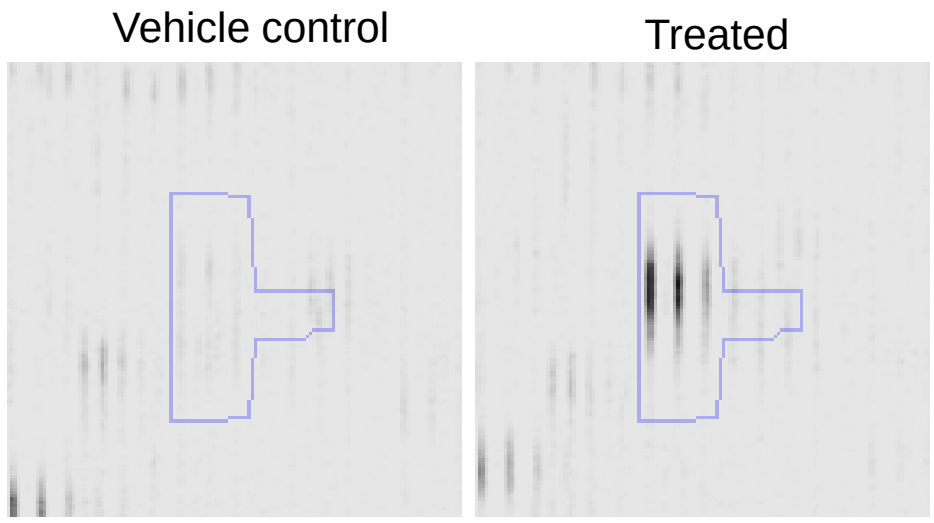


Figure 3 – Example of a differentially modulated peptide which maps to isoform 1 of Cytochrome P450 2B2.



Isoform 1 of Cytochrome P450 2B2 (IPI00193234)

Fold change = 7.5

ANOVA p-value = 1.58E-06

Mascot score = 57.55

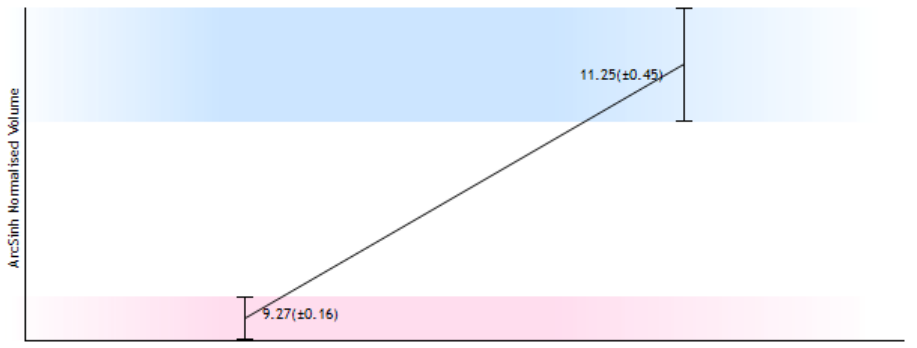


Figure 4

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%

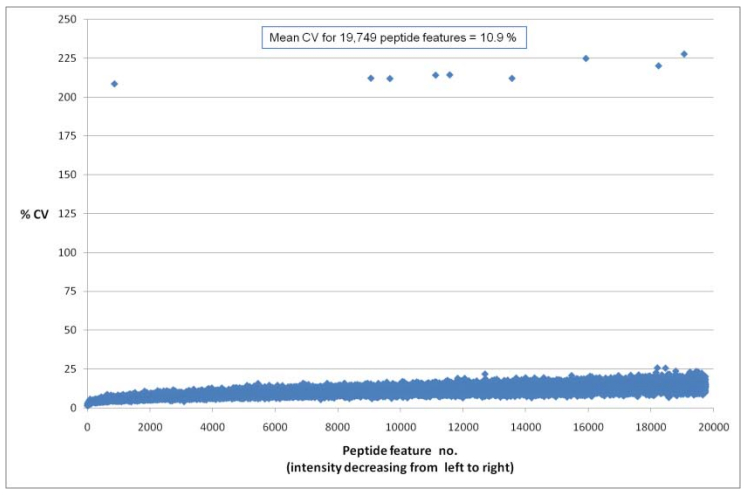


Figure 5

Volcano plot showing the fold change ratio versus ANOVA p-value for 809 identified proteins

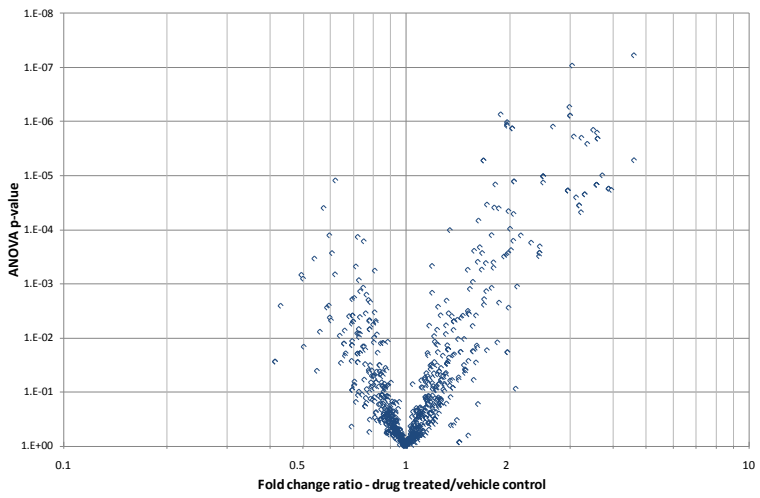


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Figure 6

Scatter plot of fold change for proteins measured by label-free LC-MS versus the corresponding mRNA measured by microarray

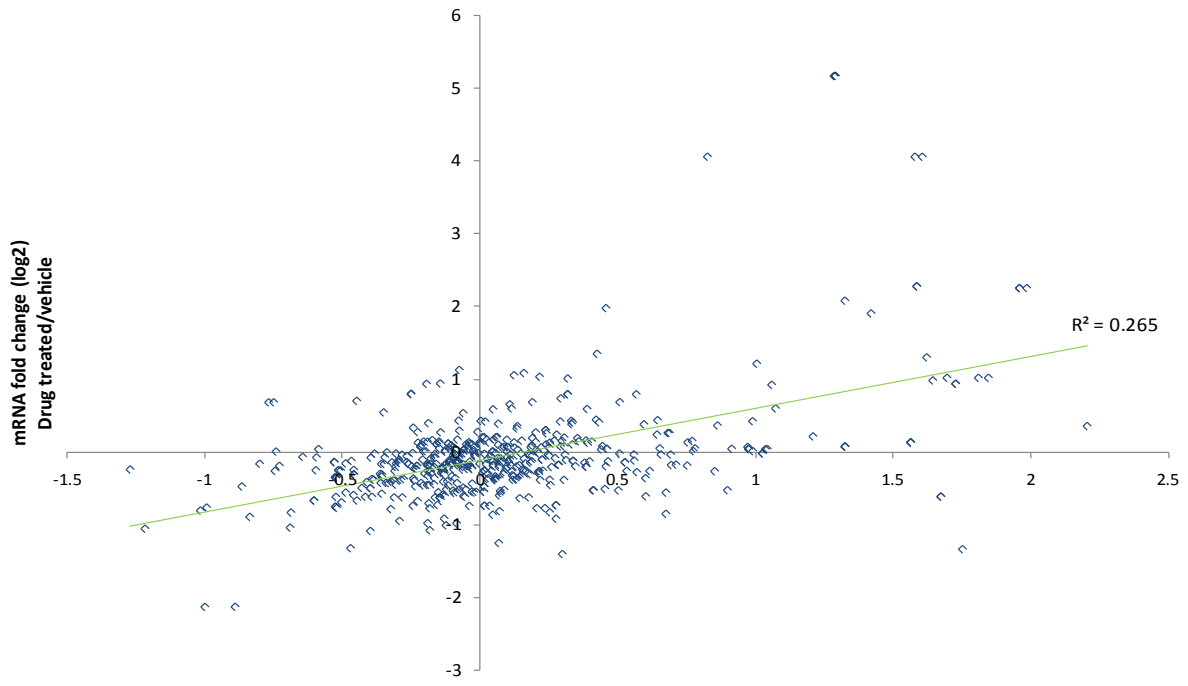
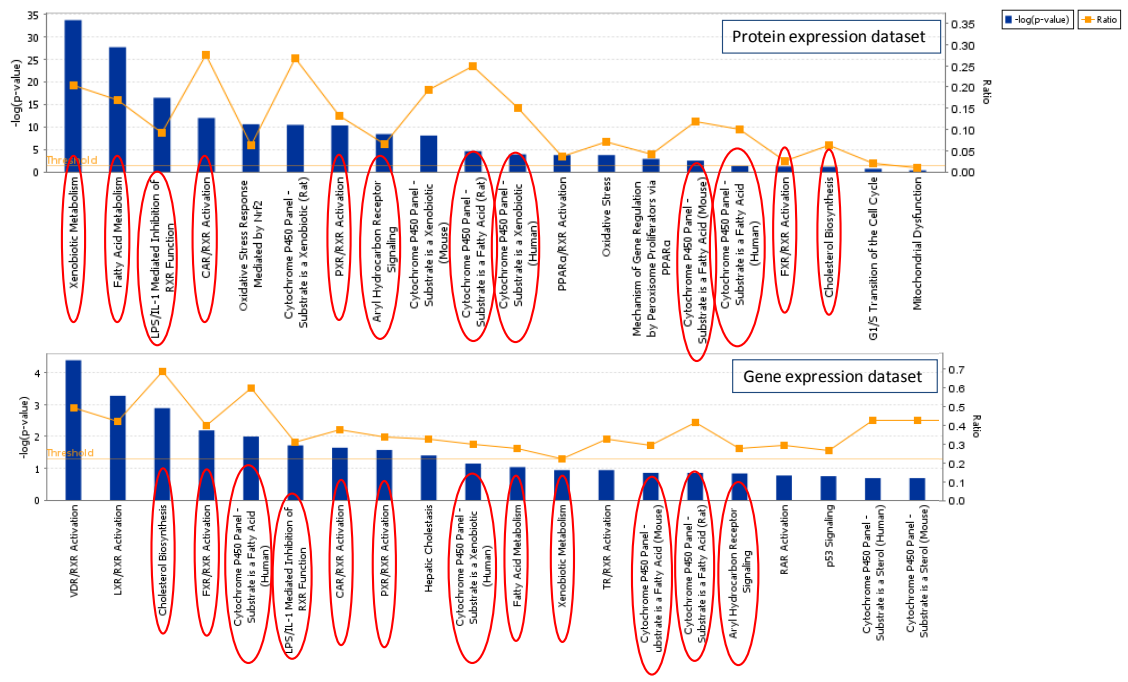


Figure 7

Comparison of Ingenuity Pathway Analyses for protein label-free LC-MS dataset (upper panel) and mRNA microarray dataset (lower panel).



Summary

- Technical variance for this label-free LC-MS method is low (mean CV = 10.9 % for 19,749 features with abundance > 1000)
- 809 proteins identified at 3.1 % false identification rate via 2,797 peptides
- The majority of peptides that are quantified are not assigned sequence annotations and so 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)
- Protein fold changes mapped to corresponding mRNA fold changes and are only weakly correlated (R^2=0.265 – see Figure 6), however, agreement at the pathway level is much more significant (see Figure 7)

Acknowledgements

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