

Quantitative assessment of whole blood RNA profiling as an early temporal marker of acetaminophen hepatotoxicity

D. Harris¹, P. Gaskin², W. Henderson¹, Y. Tessier¹, A. Templeton¹, M. Hartness¹, M. Craigon³, T. Freeman³, T. Forster³, G. Rubin⁴, A. Ivens⁴ and P. Ghazal³
¹Aptuit Ltd, ²Aptuit Consulting Inc, ³University of Edinburgh, ⁴Fios Genomics Ltd, Edinburgh, UK. Corresponding author: yann.tessier@aptuit.com



Introduction and Objectives

- Current pre-clinical safety assessment of drug candidates requires animal-, API- and resource-intensive studies. Toxicogenomics promises to identify potentially toxic compounds much earlier in development and possibly with more sensitivity and specificity than conventional methods. New analytical platforms may therefore be of use in improving compound screening and reducing drug development times and costs. Also, the ability to perform such studies using methods that are as minimally invasive to the animal would be highly beneficial both from a practical and an ethical perspective.
- Acetaminophen (also referred to as Paracetamol) is a widely used analgesic and antipyretic drug, with a well-described hepatotoxicity profile¹. This poster focuses on its hepatic toxicity. A broader and more detailed data assessment of this study is reported elsewhere². Acetaminophen was used in this study as a clinically relevant model compound for measuring hepatotoxicity.
- Recently, it has been shown that it is possible to combine early toxicological effects of acetaminophen with gene expression profiling^{3,4}. However, the pathway biology and statistical power of a systemic RNA profile from an early temporal screen for drug toxicity is still largely unknown. Our objective was to identify and quantitatively map the underlying pathway biology detectable from alterations in whole blood expression studies of the rat acetaminophen hepatotoxicity model.

Materials and Methods

- The *in vivo* phase was conducted to GLP in an AAALAC accredited animal facility. Male Sprague-Dawley rats (Charles River, UK Ltd) of approximately 5 weeks and weight range 100-124g were assigned to 4 dose-groups (0, 100, 600 and 1200mg/kg/day), each comprising n=15. Satellite groups were used for toxicokinetic evaluation (this part is not covered in this poster). The animals were dosed an acetaminophen suspension, daily by the oral route. At each dose level, 3 subgroups of n=5 were killed for whole blood toxicogenomic analysis and histopathology, respectively at 1, 3 and 14 days.
- RNA was isolated from blood using the Ambion Mouse RiboPure™ Blood RNA Isolation Kit, as per manufacturer's instructions. The quality and integrity of the RNA was analyzed on an Agilent 2100 Bioanalyzer. All samples produced the expected profile pre- and post-fragmentation. The RNA was quantified on a Nanodrop ND-1000 Spectrophotometer.
- The array platform employed for cDNA hybridization and analysis was the Affymetrix Rat 230 2.0 whole rat genome array. Samples were processed using the Affymetrix One Cycle cDNA synthesis method with a starting amount of 2 µg of RNA. This was followed by a cDNA clean-up step and an IVT step to synthesise biotin-labelled cRNA. Biotin labelled cRNA was then processed through a clean-up step prior to quantification (260/280nm) using a BioMate5 spectrophotometer.
- Samples were normalised using RNA (robust multi-chip average) which normalises data between chips. At each dose level, the inter-chip correlation was very high (>0.95).
- Normalised data were filtered to include only probesets exhibiting an IQR>0.5 log2 units; 3575 probesets remained (~12%). Pairwise comparisons were undertaken on linear model fitted data, using empirical Bayesian approaches, with correction for multiple testing. Treated samples were normalised by comparison with timed control samples. Measures of differential expression were also obtained through comparisons within a given timepoint or drug concentration series. The functional groupings of significant genes within each comparison (raw p<0.01) were determined by Gene Ontology term enrichment analysis, using a hypergeometric test (enrichment threshold = 0.001).

Results (1)

- Toxicologically**, 100 mg/kg was the NOAEL and 600 mg/kg was both the LOAEL and the MTD (at 1200 mg/kg, 1/5 rat has to be sacrificed on Day 6).
- As expected, **liver histopathology** consisted of a dose-dependent centrilobular necrosis with inflammation. With time, some findings tended to decrease in severity, as can be seen in Table 1 below.

Acetaminophen Dose (mg/kg)	Liver histopathology		
	Day 1	Day 3	Day 14
100	Normal background features of rodent liver 	Normal background features of rodent liver Centrilobular hyperplasia in 2/5 animals. 	Normal background features of rodent liver
600	Centrilobular necrosis with inflammation (up to 100%) 2/5 animals. 	Centrilobular alteration (cytotoxicic coagulation, slight necrosis) and enlargement of cells around the central vein) 2/5 animals. 	Centrilobular necrosis with inflammation (up to 100%) 2/5 animals.
1200	Centrilobular necrosis with inflammation (up to 100%) 2/5 animals. 	Centrilobular necrosis with inflammation (up to 100%) 2/5 animals. Centrilobular alteration (cytotoxicic coagulation, slight necrosis) and enlargement of cells around the central vein) 2/5 animals. 	Centrilobular necrosis with inflammation (up to 100%) 2/5 animals. Centrilobular alteration (cytotoxicic coagulation, slight necrosis) and enlargement of cells around the central vein) 2/5 animals.

Table 1 : Liver Histopathology

- No statistically significant changes in **gene expression** were observed between vehicle and the non-toxic 100 mg/kg dose, at any time point.
- Statistically significant changes in gene expression were observed between vehicle and the moderately toxic 600 mg/kg dose. After 3 days, a small number of genes showed subtle changes in expression, which increased at Day 14.
- A large number of genes showed statistically significant changes in gene expression between vehicle and 1200 mg/kg dose at all time points, with a decrease at Day 14 vs. Day 3. See Figure 1 and Table 2 over.

Results (2)

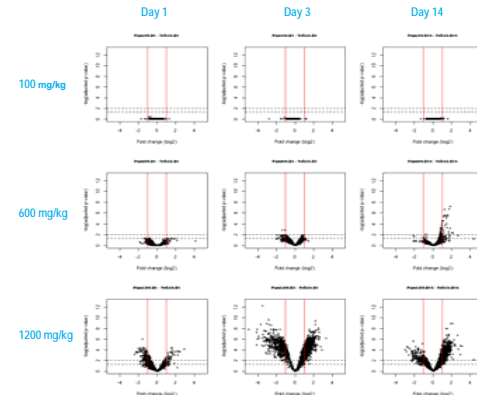


Figure 1 : Volcano plots summarising the statistical analysis. Horizontal dotted lines lower = 0.05 cut-off and higher = 0.01 cut-off. Vertical lines = 2-fold up/down regulation (Fios Genomics Ltd)

Acetaminophen Dose (mg/kg)	Time point	Gene Ontologies (GO)
100	Day 1	N/A
	Day 3	N/A
	Day 14	N/A
600	Day 1	A positive (up regulated relative to vehicle control) immune response, via MHC class II, is evident.
	Day 14	The response has become activation of the T cell receptor complex and signalling pathway.
1200	Day 1	Antigen processing via MHC class I, rather than class II, and TSD15-related ubiquitination is occurring.
	Day 3	There are similarities to the later timepoints of the 600 mg/kg terms, although in this case, the immune response is most likely to be an acute inflammatory response. Neutrophil degranulation, B cell-mediated immune response and cytotoxic activation of the JAK-STAT pathway are also suggested by the enrichment observed.
	Day 14	In addition to immune response-related terms (antigen receptor-mediated signalling pathway, T cell activation, alpha-beta T cell receptor complex), many of the up-regulated enrichment terms are related to gene expression (e.g. mRNA processing), and regulation of cell development.

Table 2 : Functional Analysis (summary)

Discussion

- In the liver, cytochromes CYP2E1 and CYP3A4 convert acetaminophen to a highly reactive metabolite which, under normal conditions, is detoxified by conjugation with glutathione. When conjugation pathways become saturated, more acetaminophen is shunted to the cytochrome P450 systems to produce the reactive metabolite, which is then free to react with cellular membranes, resulting in hepatocyte damage and death⁵.
- Others have shown the *in situ* expression profile of target organs of toxicity⁶ based on measurements in the actual tissue. A value of this work has been to establish what could be seen as the genomic signature of acetaminophen-based hepatotoxicity in a central compartment, blood. Beyond hepatic processes, genomic changes in whole blood may reflect pharmacological and/or toxicological processes occurring in many tissues (several other tissues did undergo changes), possibly including the blood itself. Other studies are probably warranted to determine what, in the genomic expression profile found, is specific of hepatic toxicity vs. what happens in other tissues. Likewise, submitting different reference compounds with various toxic mechanisms to the test could enable the establishment of an acetaminophen-type toxicity gene signature set vs. other hepatotoxicants.
- In preclinical toxicology, as shown in this study by both histopathological and genomic results, organisms have tremendous adaptive abilities. For a new molecule, translating hazards (as characterised by microarrays in laboratory animals) into risks could be challenging. Until a correlation between an expression profile and a histopathology severity can be established, the primary value of these biomarkers will be translational: early genomic findings will be of utmost interest when, based on a similar toxic mechanism in animal vs. man, they enable a safer monitoring of clinical studies.

Conclusions

- Our investigations show a statistically robust early genomic signature response to toxicological events following the administration of medium (600 mg/kg) to high (1200 mg/kg) doses of acetaminophen.
- The study has firmly established the principle of measuring, within the first two weeks, significant and detectable changes in the gene expression profiles of whole blood samples derived from the treated rats. Changes of RNA profiles are dose and time dependent, with an increase in the number and amplitude of gene expression up to Day 3 and apparently an adaptation process by Day 14.
- Overall, this study suggests that RNA biomarkers using microarray-based analyses may provide a highly reproducible platform to monitor dose and time-dependent drug responses in whole blood.

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