MULTI-OMICS ANALYSES OF FECAL EXTRACTS TO EXPLORE THE MICROBIOME OF RATS FOLLOWING ADMINISTRATION OF AN AGONIST FOR THE GPR40 RECEPTOR

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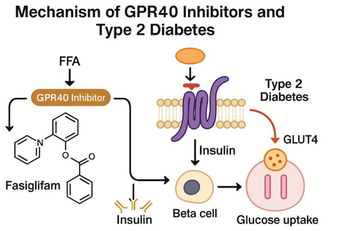
INTRODUCTION

Fasiglifam (TAK-875) is a selective, orally bioavailable GPR40 or free fatty acid receptor 1 (FFAR1) agonist, which was developed for treating Type 2 diabetes, stimulating insulin release in a glucose-dependent manner. Dose-finding studies showed that the drug significantly lowered the risk of hypoglycemia for certain patient populations. However, further data indicated safety concerns involving liver toxicity during phase III clinical trials. Previous drug metabolism studies involving TAK-875 showed that majority of metabolites reside in fecal material. Numerous studies have shown the influence of drugs on the gut microbiome and vice versa, thereby influencing drug efficacy for example. We have used a multi-OMIC approach to gain greater understanding of the effects of TAK-875 on the microbiome/biological mechanisms within a rat model.



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METHODOLOGY

The study consisted of 15 male and 9 female Sprague Dawley rats, which were acclimatized (3 days with 2 animals per cage) before being kept individually for the study duration (96 hrs). Administration of fasiglifam was either intravenously (IV; 5 mg/kg) or orally (PO; 10 or 50 mg/kg). Drug-free vehicles were also included as a control group for both IV and PO. Fecal samples were collected at four timepoints from pre-dose up to 96 hrs. Fecal samples were extracted for polar metabolites and proteins for LC-MS analyses. Polar metabolites were prepared using a folch based extraction, whilst the proteins were reduced, alkylated and tryptically digested using a filter-aided sample preparation (FASP) methodology (Figure 1).

LC-MS proteomic data were collected using the stepped quadrupole DIA methodology, SONAR Pulse (Figure 2) and further supplemented with metaproteomic data. Additionally, LC-MS metabolomic datasets were acquired using an ion mobility (IM) workflow. Data were processed (peak picked, normalized etc) prior to statistical analysis and curation for confident and stringent identifications.

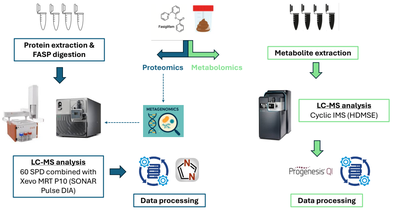


Figure 1 - Experimental workflow for the multi-OMIC analyses of fecal samples.

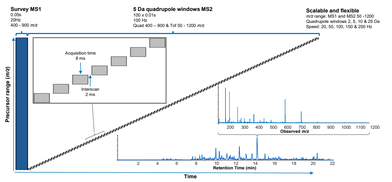
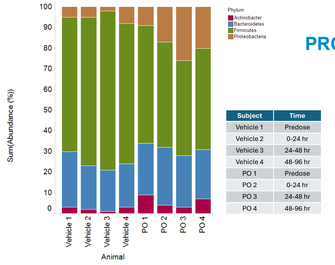


Figure 2 - SONAR Pulse DIA acquisition strategy, utilized for comprehensive proteomic profiling. Data outputted as mzML files for integration multiple informatic pipelines.

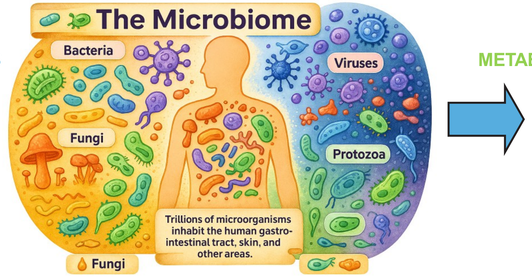
RESULTS

Proteomic data resulting from the fecal extracts provide a curated protein list >1500 identifications (unique protein ID's) from less than 10 mg of fecal material. Database searching resulted in extensive profiling of the gut microbiome (Figure 3) with four main types of phylum confidently identified. In addition to the microbiome, proteins were identified suggesting the dysregulation of numerous pathways (Figure 4) which correlated with dysbiosis and the higher levels of Proteobacteria observed from the gut microbiome. Gene Ontology (GO) processes were ordered by their significance (p-value), revealing that a high number of these processes were related to various metabolic processes and stress responses (Figure 5). Complimentary to the proteomic analyses, metabolomic/lipidomic studies (Figure 6) revealed complex small molecule profiles with expression changes for various compound classes. Statistical analysis (Figure 7) high levels of technical reproducibility but also separation for dosed animals over time. Statistical analysis also highlighted the presence of diurnal variation (Figure 8). Database searches returned identifications for those particular features but also highly correlating features, responsible for the time trajectory changes observed, which included cortisol. Cortisol correlated with some of the proteomic results, networking various identified proteins with cortisol and related compounds, predominantly involved in metabolic processes/stress response (Figure 5).

Figure 3 - Gut microbiome characterization using metaproteomics (a) for a subset of subjects analyzed (4x vehicles/4x oral dosed (PO) at 50 mg/kg) with the primary phyla being attributed to Firmicutes, followed by Bacteroidetes. These compositions vary following administration of Fasiglifam, with a general decrease in Firmicutes and to a smaller extent, Bacteroidetes. Levels of Actinobacter remain relatively constant, whilst a shift to a higher proportion of Proteobacteria is observed with dosing and time.



Subject	Time
Vehicle 1	Predose
Vehicle 2	0-24 hr
Vehicle 3	24-48 hr
Vehicle 4	48-96 hr
PO 1	Predose
PO 2	0-24 hr
PO 3	24-48 hr
PO 4	48-96 hr



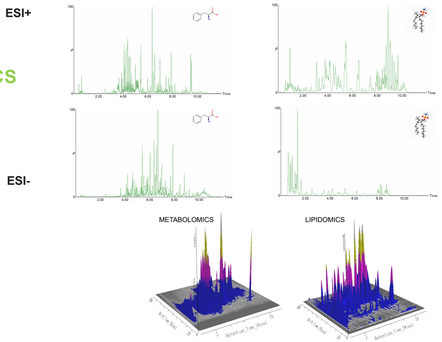


Figure 6 - Chromatograms (BPI) representing the study QC's are provided above for the metabolite (left) and lipid (right) extracts respectively. Positive and negative ionization modes are both provided. To investigate the data in three-dimensions (3D), example ion chromatograms in positive ion mode from a pooled QC sample highlight ion mobility separation and retention time, providing additional insight and interrogation of the data. These 3D plots are provided for polar metabolite and lipid-based extracts, highlighting the complexity of the microbiome.

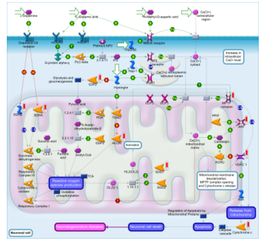


Figure 4 - Pathway mapping (Metacore) suggested mitochondrial dysfunction as a significant perturbed pathway following drug administration. Mitochondrial dysfunction plays a role with gut microbiome dysbiosis by increasing intestinal oxygen levels. This has the effect of increasing the production of Proteobacteria and therefore altering metabolic pathways and enhancing inflammation effects. Proteins identified and mapped to the pathway are indicated with red thermometers at their side.

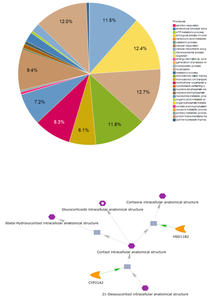


Figure 5 - Gene ontology (GO) for the top 50 processes based on ANOVA p-value (upper pie chart). A large proportion of the processes shown are linked with metabolic processes and response to stress. Cortisol was identified as highly significant compound from the metabolomics studies. The network shown here, indicates some of the identified proteins which link with the cortisol metabolic process.

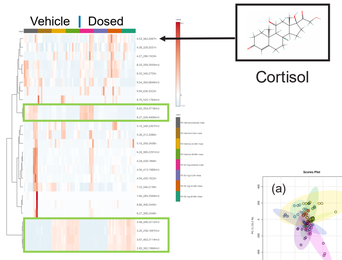


Figure 8 - Hierarchical clustering related to significant features identified between the vehicles and PO (50 mg/kg) groups. A number of the metabolites presented, show a relationship with diurnal behaviour (highlighted by green boxes). Following database searching, these features were identified with the top feature (4.53_362.2087n) being identified with high confidence as cortisol. A steroid hormone, it is well documented as being linked to stress and promoting dysbiosis—a reduction in microbial diversity and subsequently increasing harmful, pathogenic bacteria. Construction of the heatmap used Ward clustering and Euclidean Distance.

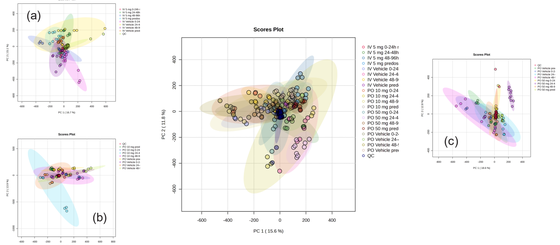


Figure 7 - Multi-variate statistical analysis for the various groups (vehicle and IV/PO dosed). These data are representative of the male group which include vehicles and 50 mg/kg dosing. The unsupervised principal component analysis (PCA) indicates a time course trajectory, which is more clearly seen with a breakdown into their respective groups (a, b & c). In particular, the plot of vehicles vs. PO (50 mg/kg) (c) indicates tight technical clustering of the study QC's (high technical reproducibility), clear separation and clustering of the 24-48 hr samples.

CONCLUSIONS

- **A multi-OMIC study investigating changes to the microbiome of the rat following administration of the GPR40 inhibitor, Fasiglifam, has been presented. Using a combination of proteomics (metaproteomics) and metabolomics/lipidomics, this has provided key insights worthy of further investigation.**
- **Metaproteomic experiments have shown changes in the phylum composition following drug administration with time. Noticeably, Firmicutes levels significantly reduce whilst Proteobacteria levels increase, suggesting potential dysbiosis, increasing inflammation and metabolic dysfunction.**
- **Complimentary metabolomic studies indicate dysregulation of a variety of compound classes (including lipids). One key metabolite which showed significant changes between vehicle and dosed groups was cortisol. This is well documented as being related to stress, promoting dysbiosis and the promotion of pathogenic bacteria.**
- **The high sensitivity and mass accuracy of the Xevo MRT P10, combined with the specificity afforded by the SONAR Pulse acquisition method allowed for comprehensive proteomic profiling of the gut microbiome, providing >1500 unique protein identifications at high confidence.**
- **Combining these proteomic data with the metabolome data, revealed potential connections between stress markers such as cortisol and increasing Proteobacteria linked with dysbiosis.**

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