LIPIDOMIC AND DESI IMAGING STUDY OF MOUSE LIVER DOSED WITH A TYROSINE KINASE INHIBITING DRUG

Nyasha Munjoma [1], Giorgis Isaac [2], Mark Towers [1], Emmanuelle Claude [1], Ian D Wilson [3], Lee A. Gethings [1], and Robert S. Plumb [2]

1 Scientific Operations, Waters Corporation, Wilmslow, SK9 4AX, United Kingdom, 2 Scientific Operations, Waters Corporation, Milford, MA, 01757, USA, 3 Department of Metabolism, Digestion and Reproduction, Imperial College, South Kensington, London, SW7 2AZ, United Kingdom

INTRODUCTION

Gefitnib is a drug belonging to a class of tyrosine kinase inhibitors (TKIs) which competes with ATP for its binding pocket in mutated or overexpressed EGFR receptors [1]. By inhibiting tyrosine kinase activity, Gefitinib prevents cancer cell proliferation. Previous plasma-based studies have shown evidence of changes in the circulating lipid composition as a result of pharmacological effects of the drug [2]. Gefitinib, N-(3-chloro-4- fluorophenyl)-7-methoxy-6-(3-morpholino-propoxy)quinazo-lin-4-amine is a drug approved in 2003 for the treatment of certain breast and non-small cell lung (NSCL) cancers as well as some other specific cancers under the brand name of Iressa®. Gefitinib acts by interrupting epidermal growth signaling in target cancer cells in the tyrosine kinase domain and is classified as an epidermal growth factor receptor (EGFR) inhibitor. Gefitinib is well absorbed in mammalian systems with good bioavailability, with peak plasma concentrations observed 3-7 hrs following dosing with a mean oral bioavailability of 60%. Gefitinib undergoes extensive biotransformation in preclinical species and humans (e.g., resulting in a large number of drug metabolites). As Gefitinib acts on the tyrosine kinase pathways and has been shown to cause liver damage, the investigation of the lipidomic effect of Gefitinib in the liver was performed [2]

HIGHLIGHTS

- **Waters CCS and RT library workflow for untargeted analyse**
- **LipidQuan™ workflow used as 'omics screen for key lipids and metabolites on Tandem Quadrupole instruments**
- **DESI imaging to interrogate spatial distribution differences**

METHODS

Nature Protocol Extraction [3]
(Dichloromethane/Methanol with SPLASH LIPIDOMIX and Gefitinib (d6) as internal standards)
- 50 mg in silica filled Eppendorf tubes
- Double extraction
- Dry and reconstitution in diluent and Odd Chain LIPIDOMIX and Gefitinib standards for Calibration curve
- Also create control, treated and study pools

Mouse liver @ pre-dose **0.5, 1, 3, 8 and 24 h**
Gefitinib was formulated in 0.5% Hydroxypropyl methylcellulose HMPC in 0.1% polysorbate 80 and administered intravenously (IV) at 10mg/kg

Untargeted Lipidomics

Column: ACQUITY™ Premier CSH C18, 2.1 x 100 mm
Mobile Phases: Acetonitrile/ Water+ Ammonium Acetate
MassLynx® Lipostar2
Data processing using LipoStar
SYNAPT ™ XS
ACQUITY™ Premier System
Additional Statistical analysis using R based package MetaboAnalyst [4]
- **8-minute run time**
- **Lipid database containing predicted CCS values de-ployed**

Targeted Lipidomics

Column: ACQUITY™ Premier BEH Amide Column, 1.7 µm, 2.1 mm X 100 mm
Mobile Phases: Acetonitrile/Water+ Ammonium Acetate
Tandem Quadrupole MS Xevo™ TQ-XS
MassLynx® Skyline
ACQUITY™ Premier System
Statistical analysis using R based package
Targeted Omics Library used to generate methods
Targeted Omics | Waters

DESI Imaging

Liver sectioned using a cryostat at a thickness of 10µm, deposited onto standard microscope slides and preserved at -80°C until analysis by mass spectrometry was performed. Additional sections used for quantification experiments

DESI™ XS

SYNAPT ™ XS

Solvent composition: 95% MeOH, 5% water with 100pg/ µL Leu-enkephalin

Flow rate: 2 µl/min
Capillary voltage: 0.65 kV
Temperature: 200°C
Nebulising gas: 10 psi
Pixel sizes: 100 µm (lateral)
Stage speed: 100 and 1,000 µm

Data processing using HDI

UNTARGETED LIPIDOMICS

The lipid sample extracts were subjected to untargeted analysis using reversed – phase LC-IM-MS(TOF) analysis. Sample extracts were loaded onto a 2.1 x 100mm 1.7µm C18 column and eluted with ammonium acetate buffered aqueous acetonitrile : acetonitrile/isopropyl alcohol gradient over 8 minutes with both positive and negative ion ESI detection following ion mobility separation. The initial resulting positive and negative ion ESI data was subjected to peak feature detection using Progenisis QI™ software prior to transfer to MetaboAnalyst [4] for statistical analysis using pareto scaling. The PCA and PLS-DA analysis score plots of the positive ion ESI unnormalized positive ESI data are given in **Figure 1**. In the unnormalized data the Pc1 and Pc2 accounted for 41% and 19.6% of the variation observed in the data, for the normalized data Pc1 and Pc2 accounted for 44% and 21.2% of the variation observed in the data. The resulting statistical data shows that the dosed and vehicle only samples were clearly separated from each other. The study reference QCs were positioned in the centre of the dosed group for both methods of statistical analysis, with the clustering slightly tighter with the PLS-DA data sets.

Figure 1. PCA(A) and PLS-DA (B) multivariate statistical model of raw positive Ion HDMSe data from Progenesis QI. Peak intensity, excluding variables with less than 50%, Interquantile Range Filter (IQR) and pareto scaling were used (Standards, Gefitinib and metabolites included).

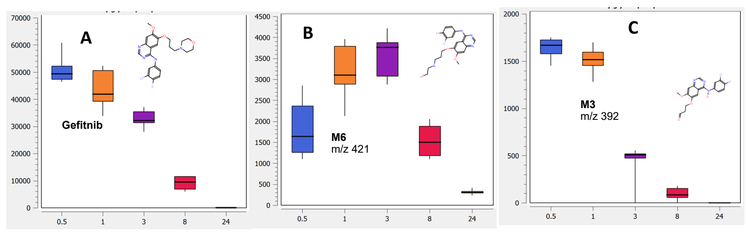


Figure 2. Gefitinib and metabolites M6 and M3 resulting from the HDMSe analysis.

LipoStar™ was used to process the dosed samples. Gefitinib and some metabolites previously detected in plasma samples [2] were detected **Figure 2**. PLS-DA models of the negative and positive ion ESI data from the dosed group only showed separation of the time points. There was an observable time related trajectory from the 0.5 – 1-h time-point, through the 1 – 3- h and 3 – 8- h sampling occasion with the 8 – 24- h samples clustering close to the 0.5 – 1- h samples, **Figure 3 and 4**. The top 25 contributing features after removal of gefitinib and its metabolites, with the highest VIP scores, were searched against an in-house constructed database using t_R, *m/z*, and the collision cross section (CCS) from the IM separation to create a list of potential identifications. These potential lipid identifications were compared, where possible, to authentic standards. Some key lipids were also analysed using the targeted lipidomics methodology.

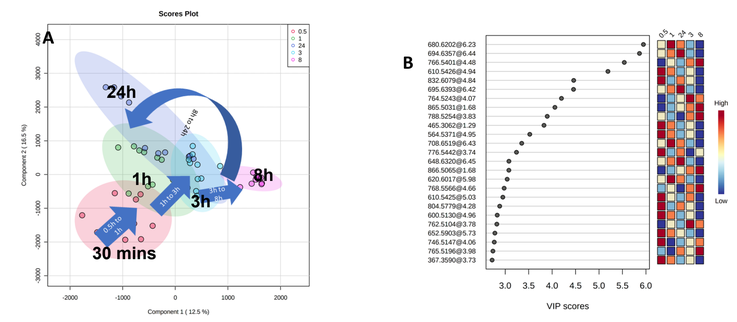


Figure 3 PLS-DA of negative ion ESI data excluding control/pre-dose. Peak intensity, excluding variables with less than 50%, Interquantile Range Filter (IQR) and pareto scaling. (LIPIDS ONLY -Gefitinib and metabolites EXCLUDED) .(A) Score Plot and (B) Top 25 variable importance in projection (VIP).

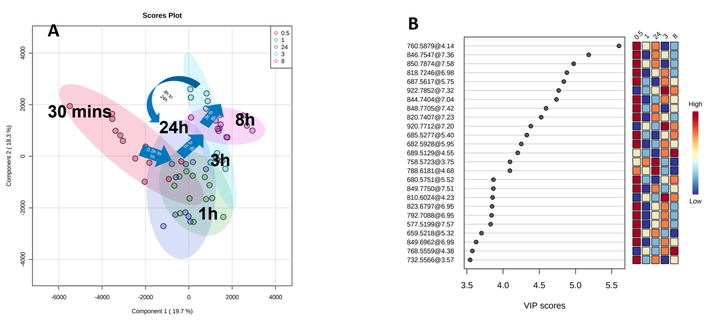


Figure 4. PLS-DA of positive ion ESI data excluding control/pre-dose. Peak intensity, excluding variables with less than 50%, Interquantile Range Filter (IQR) and pareto scaling. (LIPIDS ONLY -Gefitinib and metabolites EXCLUDED). (A) Score Plot and (B) Top 25 variable importance in projection (VIP).

TARGETED LIPIDOMICS

LipidQuan™ is a rapid HILIC based liquid chromatography tandem mass spectrometry (LC-MS/MS) platform that can be implemented to develop targeted assays. Method development and training costs are reduced when using the Quanpedia™ Library which contains over 2000 lipids with improved identification and specificity (*e.g.*, the use two fatty acyl chain fragments for phospholipid related MRMs increases specificity). In this study over 500 biologically relevant lipids were deployed to screen tissue samples. The methodology provides a broad coverage of multiple lipid classes as well as facilitating the measurement of low abundance bioactive lipids such as PAs, PSs, and PCs. We have applied the LipidQuan methodology to the analysis of mouse liver tissue following the intravenous (IV) administration of Gefitinib, an inhibitor of epidermal growth factor receptor's (EGFR) tyrosine kinase domain which is used to treat various cancers. The resulting data showed dysregulation of the lipid metabolism pathways with a time-related trajectory following the administration of the drug. For a more detailed description see, LipidQuan™: A Robust LC-MS/MS Methodology for Rapidly Profiling the Lipidome of Liver Tissue Following Metabolism of the Drug Gefitinib | Waters

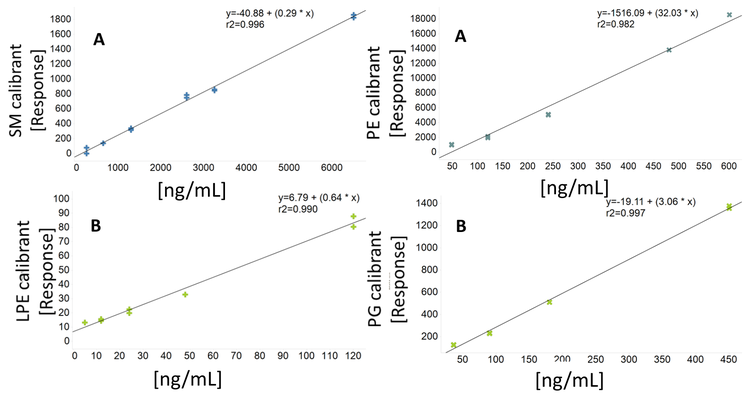


Figure 5. Example calibration curve from positive mode screening SM (A) and LPE (B) standards from Avanti Odd Chain Mix used as calibrants.

Figure 6. Example calibration curve from negative mode screening PE (A) and PG (B) standards from Avanti Odd Chain Mix used as calibrants.

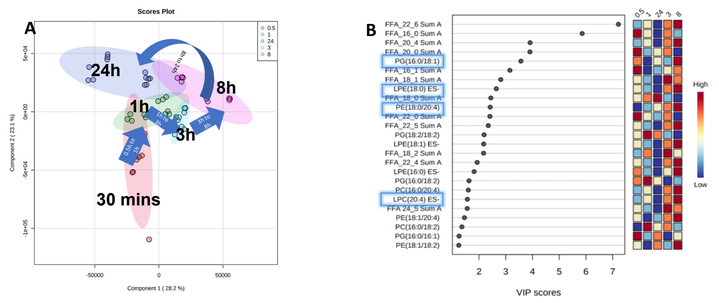
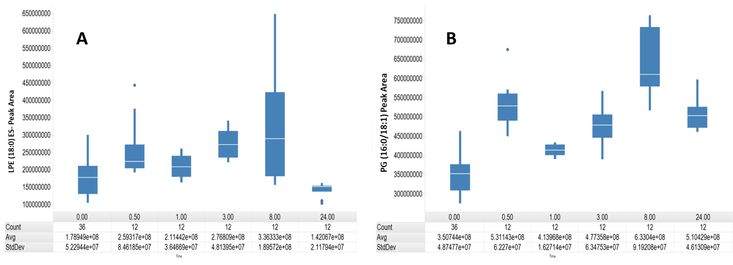


Figure 7. PLS-DA of negative ion Omics MRM screening. Excluding control/pre dose. Peak intensity, excluding variables with less than 50% and pareto scaling. (LIPIDS ONLY -Gefitinib and metabolites EXCLUDED) (A) Score Plot and (B) Top 25 variable importance in projection (VIP).



	0.00	0.50	1.00	3.00	8.00	24.00
Count	36	12	12	12	12	12
Avg	1.78949e+08	2.59317e+08	2.11442e+08	2.76809e+08	3.36333e+08	1.42067e+08
StdDev	5.22944e+07	8.48185e+07	3.64666e+07	4.81355e+07	1.89572e+08	2.11794e+07

	0.00	0.50	1.00	3.00	8.00	24.00
Count	36	12	12	12	12	12
Avg	3.50744e+08	5.31143e+08	4.13968e+08	4.77356e+08	6.33046e+08	5.10429e+08
StdDev	4.87477e+07	6.227e+07	1.62714e+07	6.34753e+07	9.19208e+07	4.61329e+07

Figure 8. Box and whisker plots of selected VIP lipids from negative ion Omics MRM screening (Figure 7) across the various timepoints (A) LPE(18:0) (B) PG(16:0/18:1)

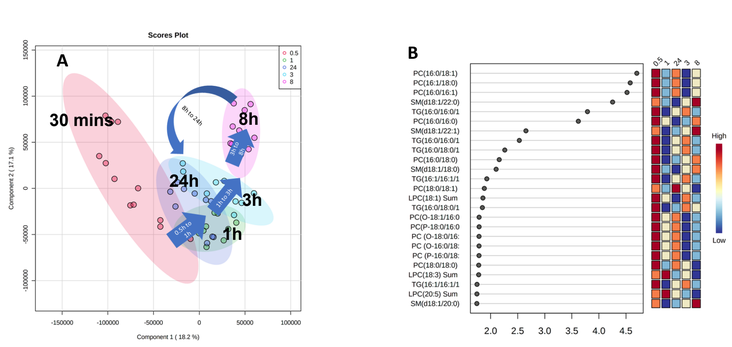


Figure 9. PLS-DA of positive ion Omics MRM screening excluding control/pre-dose. Peak intensity, excluding variables with less than 50% and pareto scaling. (LIPIDS ONLY -Gefitinib and metabolites EXCLUDED). (A) Score Plot and (B) Top 25 variable importance in projection (VIP).

DESI IMAGING

Desorption Electrospray Ionization Mass Spectrometry (DESI) imaging produces label-free, multiplexed and objective measurement of molecular targets from complex surfaces. This direct-from-sample analytical technique provides researchers with the spatially resolved molecular information needed to quickly and objectively interpret molecular profiles and understand mechanistic insights with confidence. DESI is a soft ionisation technique performed under ambient environmental conditions and requires no sample preparation.

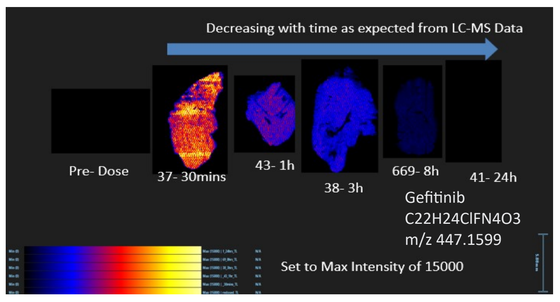


Figure 10. DESI imaging of study tissues confirmed the LC-MS findings and distribution of Gefitnib in the tissue at the various timepoints.

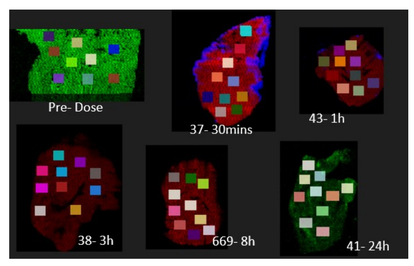


Figure 11. DESI imaging can also be used to perform multivariate statistics by collecting spectra across the tissue called Regions of Interest (ROI). These spectra can then be grouped and analysed using statistical packages such as MetaboAnalyst as shown in Figure 12.

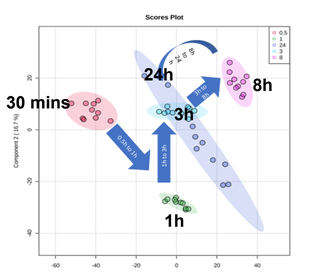


Figure 12. PLS-DA score plot of positive ion DESI image ROI. Excluding control/pre-dose. Peak intensities used, excluding variables with less than 50% and pareto scaling.

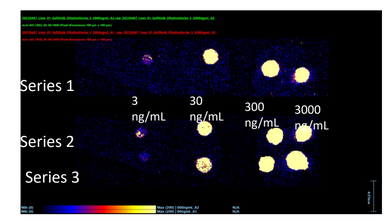


Figure 13. DESI imaging can also be used to determine concentrations by spiking known concertation of standards on to tissue and constructing calibration curves as shown in Figure 14 by collecting spectra across the tissue.

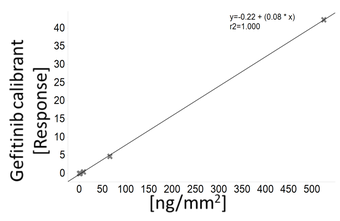
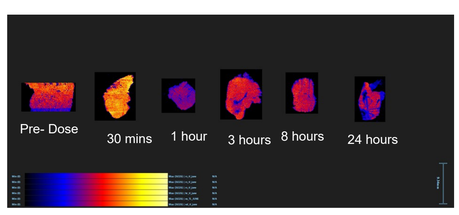


Figure 14. Example calibration curve of Gefitinib standards using DESI. The response is measured against the ng/mm^2.



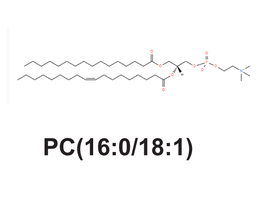


Figure 15. DESI Image using the distribution of PC(16:0/18:1) which was shown to be the top VIP following multivariate statistics of both untargeted and targeted LC-MS experiments (Figure 4 and Figure 9).

CONCLUSION

- **Comprehensive lipidomic studies were performed using a small amount of tissue sample by implementing the described workflows**
- **Time-related trajectories of lipid biosynthesis was observed using the various workflows even after exclusion of the drug and its metabolites**
- **Key lipids that contributed to multivariate statistical models were conserved across the lipid analysis platforms**
- **Predicted CCS values help improve confidence of lipid identifications when performing discovery or untargeted experiments**
- **LipidQuan provided a fast and effective method for the analysis of biological fluids and tissue samples**
- **DESI imaging has great potential for the interrogation of the spatial distribution of drugs and lipids in tissue samples**

References

1. McKillop D, Hutchison M, Partridge E.A, Bushby N, Cooper C.M, Clarkson-Jones J.A, Herron W, Swaisland H.C. Metabolic Disposition of Gefitinib, an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, in Rat, Dog and Man. Xenobiotica 2005, 34, 914-934
2. 1.Molloy B.J, King A, Mullin L.G, Gethings L.A, Riley R, Plumb R.S, Wilson I.D. Rapid Determination of the Pharmacokinetics and Metabolic Fate of Gefitinib in the Mouse Using a Combination of UPLC/MS/MS, UPLC/QToF/MS, and Ion Mobility (IM)-enabled UPLC/QToF/MS. Xenobiotica 2021, 51, 434–446
3. 1.Want, E., Masson, P., Michopoulos, F. et al. Global metabolic profiling of animal and human tissues via UPLC-MS. Nat Protoc, 2013, 8, 17–32. https://doi.org/10.1038/nprot.2012.135
4. 1.Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucl. Acids Res., 2018, 46, W486-494.