Lipidomics Discovery Profiling and Targeted LC/MS Analysis in 3T3-L1 Differentiating Adipocytes

Application Note

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Abstract
The mechanism(s) underlying the formation of adipose tissue is of tremendous scientific interest due to the potential to mitigate obesity. The 3T3-L1 cell line has been a valuable model for studying this process since many of the molecular processes that drive differentiation of this cell line in vitro is consistent with the processes of adipogenesis in vivo. To gain greater insight into these lipid changes, Q-TOF LC/MS was used for lipidomics discovery profiling in both untargeted and targeted modes to find potential lipid biomarkers of differentiation in adipocytes.

Introduction
Lipidomics is a branch of metabolomics and is a systems-based study of all lipids (non-water soluble metabolites), the molecules with which they interact, and their function within the cell. Lipid abnormalities contribute to many diseases, including atherosclerosis, diabetes, obesity, Alzheimer's and metabolic disease.

The differentiation of 3T3-L1 cells has previously been extensively studied using a variety of approaches, including microarray and protein expression analyses [1-5]. These different approaches all provide important yet complementary information. However, there is very limited LC/MS lipid profiling information available for this cell line that reveals the important changes that occur in some of the major lipid families during differentiation.

An LC/MS system composed of an Agilent 1200 Series LC and Agilent 6520 Quadrupole Time-of-Flight (Q-TOF) LC/MS was selected for this study due to the high retention time reproducibility, sub-2 ppm mass accuracy and outstanding abundance reproducibility necessary for successful profiling experiments. Agilent MassHunter Qual, MassHunter Mass Profiler and GeneSpring-MS bioinformatics software was used to analyze the complex, multi-class data generated by this study.

Differentiation in 3T3-L1 cells is an 8-day process that is initiated by the addition of a hormone cocktail to pre-adipocytes. This study focused on profiling lipids at days 0, 2, and 8 during the differentiation process to find and identify metabolites whose abundances significantly changed over this time period. For each time point three biological sample replicates were analyzed. These results reveal that the metabolite profiles, composed of many lipid species were able to clearly distinguish between the different time points. In addition, many of the changing lipids followed patterns expected for the formation of adipose cells.
LC/MS Analysis

MS only data was acquired in both positive and negative ion modes using an Agilent 1200 Series LC and Agilent 6520 Quadrupole Time-of-Flight (Q-TOF).

Negative ion mode runs

Column: Gemini C18 reversed phase column (3.5 µm, 4.6 x 50 mm)
Guard: C18 reversed phase (3.5 µm, 2 x 20 mm)

Mobile Phase A – 95:5 water : methanol 0.1% ammonium hydroxide
Mobile Phase B – 65:30:5 isopropanol : methanol : water 0.1% ammonium hydroxide

Gradient:
0% B at 0 min
20% B at 5 min
100% B at 65 min
0% B at 85 min

MS stop time: 95 min
LC stop time: 95 min
Column temperature: 30°C
Flow rate: 0.4 mL/min (0.1 mL/min 0 to 5 min)
Injection volume: 30 µL

Positive ion mode runs

Column: Luna C5 reversed phase column (3.5 µm, 4.6 x 50 mm)
Guard: C4 reversed phase (3.5 µm, 2 x 20 mm)

Mobile Phase A - 95:5 water : methanol 0.1% formic acid, 5 mM ammonium formate
Mobile Phase B - 65:30:5 isopropanol : methanol : water 0.1% formic acid, 5 mM ammonium formate

Gradient: same as negative mode

MS conditions

MS System: Agilent 6520 Quadrupole Time-of-Flight (Q-TOF) LC/MS
Ionization mode: ESI
Ionization Polarity: Negative and Positive
Drying gas flow: 10 L/min
Drying gas temperature: 350°C
Nebulizer pressure: 45 psi
Data range: 100-1,500
Acquisition Rate: 1020.4 ms/spectrum
Fragmentor voltage: 100 V
Skimmer voltage: 60 V
Octopole RF voltage: 250 V
Capillary voltage: 3,500 V
Reference masses (m/z):
  negative ion 119.0363, 980.016375
  positive ion 121.0509, 922.0098

Results and Discussion

Analysis of the 3T3-L1 cells lipidome by LC-MS identified many metabolomic changes between pre-adipocytes (day 0) and mature adipocytes (day 8), using both non-targeted (i.e. discovery profiling) and “targeted” (using a list of known compound formulas) approaches. The day 2 cells, transitioning to become adipocytes, could also be differentiated based on their abundance profile differences from the earlier and later time points. In our untargeted analyses, the data analysis workflow we employed
Thus, for our targeted approach, we constructed a METLIN Personal Metabolite database of 179 lipid compounds that are believed to be involved in adipocyte differentiation. We then queried each sample data file in MassHunter Qual by loading the lipid database containing the formula for each lipid species. Each formula was subsequently used to query the data files using a “Find by Formula” function.

(Fig. 2) included pairwise comparisons of pre-adipocytes (day 0) and mature adipocytes (day 8) in Mass Profiler software, as well as PCA, 1-way ANOVA and K-means cluster analysis in GeneSpring MS software that differentiated the cells at various stages of adipogenesis.

The acquisition of spectral data on a Q-TOF instead of targeted multiple reaction monitoring (MRM) data on a triple quadrupole (QQQ) permits both non-targeted and targeted data analysis from a single analytical run. Thus, for our targeted approach, we constructed a METLIN Personal Metabolite database of 179 lipid compounds that are believed to be involved in adipocyte differentiation. We then queried each sample data file in MassHunter Qual by loading the lipid database containing the formula for each lipid species. Each formula was subsequently used to query the data files using a “Find by Formula” function.

Untargeted Metabolite Identification

1. Pairwise analysis of day 0 and day 8 data sets

After performing Molecular Feature Extraction (MFE) of all data files in MassHunter Qualitative Analysis software, the resulting .mhd files were imported into Mass Profiler software for statistical analysis and identification. The files were aligned and binned using specified mass and retention time (RT) tolerance windows. A pairwise analysis of pre-adipocytes (day 0) and mature adipocytes (day 8) was performed for the triplicate samples per group. A plot of Mass and RT revealed several interesting features about the data acquired in ESI (+) mode, including a pattern consistent with a single –CH₂ subunit in growing (or degrading) polymeric chains, spaced at approximately 0.5 min intervals. These polymers appeared only in day 8 adipocytes, starting at approximately 55 min in the LC/MS analysis (Fig. 3A). A plot of the abundances of 1,532 features on the log₂ scale revealed many differential metabolites with > 4 fold change, particularly in the direction of mature adipocytes (Fig. 3B).
Grouping and filtering of high quality features
A. Triplicate ESI (+) processed .mhd files for each day were imported into GeneSpring MS software
B. Total number of binned and aligned features = 10,011
C. Group (i.e. average) the triplicate sample abundances by day
D. Apply a frequency filter: retain only those features present in all triplicates in at least one group (day) = 2,108
E. Apply error filter on (D) above: retain features having %CV in the range from 0 to 1,000 = 1,209

Statistical Analysis
A. Perform principle component analysis (PCA) on 1,209 features (i.e. “high quality”) set (E)
B. Perform analysis of variance (ANOVA) on (E) above, p < 0.05 with Tukey post-hoc test for days 0, 2 and 8
C. K-Means cluster analysis on (E) above to identify groups of co-varying masses across the 3 days

In order to identify the metabolites, the METLIN Personal Metabolite Database was subsequently queried directly from within Mass Profiler software. Fig. 4 shows a selected summary of proposed compounds based on METLIN database matches for day 8 vs day 0 3T3-L1 cells. Also included in the table is the log2 ratio and an associated p-value (Student’s t-test) of significance based on triplicate samples. Missing values were treated as being 0.

2. Multi-group analysis of day 0, day 2 and day 8 data sets
GeneSpring MS software was used for the discovery of metabolite biomarkers through the analysis and visualization of LC/MS data. A comprehensive suite of powerful statistical analysis tools were used to profile metabolites associated with changes in lipidogenesis function, enabling the rapid discovery of these potential biomarkers. The following workflow was used for untargeted metabolite identification in GeneSpring MS software:

PCA analysis of the filtered data set (i.e. 1,209 masses) revealed patterns showing clear separation of the triplicate samples based on the abundance profiles of those metabolites across the three days (Fig. 5).
In addition, due to the large number of differential metabolites, we also performed K-means cluster analysis that identified pools of co-regulated compounds (Fig. 6).

We analyzed several clusters, including those that increased in day 8 (cluster 4), decreased in day 8 (cluster 6), or were transiently elevated in day 2 (cluster 7) during differentiation.

Within these clusters, each of the target masses identified in the profiling processes was searched against the METLIN database. The database was searched over a 10 ppm mass window. The empirical formula calculation was set to a mass error window of 5 ppm. With this analysis, many different classes of metabolites (predominantly lipids) were identified as significantly changing throughout the differentiation process. For example, Fig. 7 shows a METLIN search result for cluster 4, revealing several lipids in day 8 adipocytes to be elevated.

Separation in negative ion mode was also observed (not shown). An ANOVA with a Tukey post-hoc test provided a list of significantly differential compounds during differentiation of 3T3-L1 cells, and they were identified by matching to the METLIN database. In addition, due to the large number of differential metabolites, we also performed K-means cluster analysis that identified pools of co-regulated compounds (Fig. 6).

![Figure 6. K-Means cluster analysis in GeneSpring MS software. Several clusters are highlighted by red boxes (4, 6 and 7), having different relative metabolite abundance profiles for day 8 adipocytes relative to day 2 and day 0 3T3-L1 cells.](image)

![Figure 7. A summary of compound identification search results for cluster 4 using the METLIN database.](image)
Targeted (guided) metabolite identification

A list of known lipid compounds was created from several different lipid classes (Table 1) and used to construct a custom METLIN Personal Metabolite Database of over 170 lipids. The custom built lipid database was then used to interrogate the sample data files from each ESI acquired ion polarity in a “targeted” or guided fashion in MassHunter Qual. This was accomplished using the “Find by Formula” algorithm, which finds and extracts all the co-eluting isotopes, salt adducts, dimers, etc. belonging to each empirical formula. Several classes of metabolites were identified this way. These included lipids such as triglycerides, odd chain free fatty acids, and phosphatidyl choline. Interestingly, many of the changing species contained an acyl chain in their structure that is also consistent with the non-targeted, discovery analysis results that was observed in Fig. 3A.

The importance of fatty acyl CoA oxidation in reshaping the acyl chain landscape throughout differentiation has previously been defined [7]. Those observations are consistent with the results shown here where the changes in acyl chains cause a shifting distribution of acylated species such as triglycerides. For example, while the entire pool of triglycerides is greatly increased during differentiation, specific species changes such as trioleoyl glycerol are actually lower at day 8 while species containing shorter acyl chains, a product of fatty acyl CoA oxidation, are strongly elevated. Furthermore, increases in odd chain acyl groups in glycerophospholipids, such as C33:2 PC, is expected due to an increase in the rate of alpha oxidation in differentiating 3T3-L1 cells [7].

The importance of these changes is not currently known but the ability of metabolomics approaches to discover such changes will likely lead to a better understanding of the differentiation process. Finally, the results from all METLIN searches for all clusters were incorporated into Excel spreadsheets for further interpretation. This enabled the selection of individual lipids that were differential across the three days, and to retrieve their profile information in GeneSpring MS software (Fig. 9).

Table 1. List of lipid families identified in the 3T3-L1 cell line that was used to create a list of compounds in METLIN database for targeted profiling.

<table>
<thead>
<tr>
<th>Free fatty acids</th>
<th>Sphingomyelins</th>
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<tbody>
<tr>
<td>Monoacylglycerols</td>
<td>Phosphatidylcholines</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>Phosphatidylethanolamines</td>
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<tr>
<td>Triacylglycerols</td>
<td>Phosphatidylserines</td>
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<tr>
<td>Cholesterol</td>
<td>Phosphatidylinositosls</td>
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<tr>
<td>Cholesteryl esters</td>
<td>Phosphatidylglycerols</td>
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<tr>
<td>Steroid hormones</td>
<td>Phosphatidic acids</td>
</tr>
<tr>
<td>Retinoids</td>
<td>Lysophospholipids</td>
</tr>
<tr>
<td>Ceramides</td>
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</tbody>
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Figure 8. Temporally regulated metabolites were identified using GeneSpring MS software. C18:1 acyl carnitine was found to be elevated by K-Means analysis at day 2 compared to day 0 and day 8.

Figure 9. GeneSpring MS software results showing elevated normalized abundance profiles of day 8 samples for (A) m/z 228.2078 and (B) m/z 254.2233, corresponding to C14H28O2 (myristic acid) and palmitoleic acid (C16H30O2), respectively.
In positive ion mode, decreased abundance of an m/z 884.78329 species matching that of TAG_C54:3 was observed during differentiation (Fig. 10A). This is consistent with generalized acyl chain shortening during differentiation due to increased protein levels and activity of stearoyl-CoA desaturase [2]. Furthermore, a dramatic, concomitant increase in the levels of m/z 804.72069, matching the formula for TAG_C48:1 (Fig. 10B), was also observed, and contains at least one degree of unsaturation.

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In negative ion mode, the rate of alpha oxidation increases during differentiation resulting in the appearance of odd chain free fatty acids (Fig. 11).

Indeed this pattern was observed for m/z 268.24023, corresponding to the formula for odd chain Free Fatty Acid C17:1 (OddFFA_C17:1) in day 8 samples only (Fig. 12).
Conclusions

We have demonstrated the capabilities of a complete suite of Agilent software for performing differential lipidomics profiling in the context of LC/MS discovery workflows in both untargeted and targeted modes. By incorporating both fast and robust peak finding algorithms, differential analysis programs and visualization software, we were able to show that the lipid profiles of these cells could be clearly distinguished between day 0, 2, and 8 of differentiation. Moreover, many lipid compounds were found to have matches to the METLIN and LipidMaps databases. GeneSpring MS analysis provided valuable information through PCA as well as the application of the K-means algorithm to identify metabolites that respond in a similar fashion. This was instrumental in identifying a number of important lipid classes and revealed underlying changes in metabolism related to fatty acid oxidation, which impacted all acylated species. In addition, a series of transiently elevated metabolites were discovered, and these lipids will provide a starting point to help understand the metabolic changes that occur in the transition from preadipocytes to adipocytes. Lastly, these same metabolites can be analyzed in a variety of different systems including human samples.

References


