Sphingolipids (and precursor fatty acyl-CoA’s)
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Other LIPID MAPS Sphingolipid Core members:

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<th>Mass spectrometry</th>
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<td>Jeremy Allegood</td>
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Outline:

A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze
B. Sample preparation issues: solvents, chromatography, recovery, reproducibility
C. Compound identification: Characteristic fragmentations; MS/MS and MS^n (LC for isomers and isobars, etc.)
D. Quantitation: MRM, Internal standards, etc.
E. Data analysis/visualization: LIMS, Website, other
F. Remaining challenges and opportunities
G. Discoveries from sphingolipidomic analysis thus far
H. Comparison of Lipid MAPS methods with others in the literature
A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze

**Backbone variation**

**Sphingoid base:**
- Sphinganine (d18:0)
- 4-Hydroxysphinganine (phytosphingosine) (t18:0)
- D-erythro-sphingosine (d18:1)

**Ceramide:**
- Shown: N-palmitoylsphingosine (d18:1/16:0)
  - Other fatty acids - typically C16-C26
  - 0-1 double bond
  - sometimes α-hydroxy

**Headgroup variation**

**Phosphosphingolipids:**
- -OP(O₂⁻)O-choline, Sphingomyelin

**Glycosphingolipids:**
- Glc, Gal, Lac, Sulfatides…>400; see www.lipidmap.org; www.sphingomap.org

- Lactosylceramide (LacCer)
- GalNAc III
- Gal IV
- NeuAc
- Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glc1-1Cer
  - II³Neu5AcGg₄Cer
  - GM3
B. Sample preparation issues: solvents, chromatography, recovery, reproducibility:

A. (Polar)

1. 30-100 mm Petri dish w/cells
2. PBS scrape pellet
3. + CH₃OH + CHCl₃ + Internal Stds (Avanti)
4. Sonicate 48 °C ~12 h
5. 0.1 M KOH 37 °C ~2 h
6. Dry, Redissolve in LC mobile phase
7. Centrifuge & transfer to autoinjector vial
8. Reverse phase LC-MSⁿ >80%

B. (Non-polar)

1. + HOAc + CHCl₃ + H₂O
2. Centrifuge & Recover Lower phase
3. Dry, Redissolve in LC mobile phase
4. Centrifuge & transfer to autoinjector vial
5. Normal phase LC MSⁿ 80-90%
C. Compound Identification: Characteristic fragmentations of sphingolipids

\[ \text{R} = \text{n-alkyl chain}, \text{R'} = \text{H, Glu/Gal, Lac}, \text{and R''} = \text{H, PO}_3 \]
Sphingolipid analysis by LC-MS/MS

A (Polar, e.g. So, Sa, 4-HO-Sa, SoP, So-Pcholine, Psychosine, Cer-P, etc.)

B (Nonpolar, e.g. Cer, Cer-P, SM, GlcCer, GalCer, LacCer, etc.)

Normal phase HPLC

Reverse phase HPLC → Inlet System → Ion Source → Mass Analyzer (Q1, Q2, Q3 or LIT) → Detector

Species analyzed to date (in most mammalian sphingoid base & FA variants):

✓ SM’s, GlcCer, GalCer, LacCer, Sulfatide (quantitative analysis of globosides and gangliosides)
✓ Cer & Cer-P
✓ So, Sa and 4-hydroxy-Sa
✓ So-P, Sa-P and other derivatives (lysoSM, psychosine, N-methyl-)
✓ Plus metabolites labeled with stable isotope precursors

Data System for quantitation by MRM (Multiple Reaction Monitoring) w/ appropriate internal standards

Sullards, Merrill & coworkers
Summary of conditions used for Liquid Chromatography

**Reversed Phase**
- LCB analysis: So, Sa, phyto So, So-1-P, Sa-1-P, and standards
- 2.1 x 50mm Supelco Discovery C18, 5 µm, 120 Å
- A: 74:25:1 CH₃OH/H₂O/HCOOH
  - B: 99:1 CH₃OH/HCOOH
- Flow rate 1 mL/min, 0.6 min. 80:20 A/B, 1.8 min. to 100% B, 0.6 min. hold 100% B

**Normal Phase**
- Complex SL's: Cer, GlcCer, GalCer, LacCer, SM, and standards
- 2.1 x 50 mm Supelco NH₂, 3 µm, 120 Å
- A: 97:2:1 CH₃CN/CH₃OH/CH₃COOH
  - B: 99:1 CH₃OH/CH₃COOH
  - both 5mM Ammonium Acetate
- Flow rate 1.5 mL/min, 0.5 min. 100% A, 0.2 min. gradient to 90:10 A/B, 0.5 min. hold, 0.4 min. to 82:18 A/B, 0.6 min hold, 0.4 min. to 100% B

Approx. number of molecules per RAW cell (million)

Serine + Palmitoyl-CoA → 3-Ketosphinganine → Sphinganine (d18:0) → Sphinganine 1-phosphate (d18:0-P)

CH$_3$(CH$_2$)$_{10}$CH$_2$ \[\text{NH}_3^+\]

11.7

Ceramide synthases + Fatty acyl-CoA

All subspecies have been quantified for these chain lengths, too (only a few are shown; all are at www.lipidmaps.org)

Sphinganine 1-phosphate (d18:0-P) → Ceramide synthases + Fatty acyl-CoA

Approx. number of molecules per RAW cell (million)

LacCer <0.1

Sulfatide <0.1

3-Ketosphinganine

Sphinganine (d18:0)

Sphinganine 1-phosphate (d18:0-P)
Variation in backbone, acyl chain & headgroup specificity

Sphinganine (d18:0)

H

CH

2

OH

HO

NH

3

(+)

CH

3

(CH

2)

10

CH

2

3-Ketosphinganine

Sphinganine 1-phosphate
(d18:0-P)

Ceramide synthases + Fatty acyl-CoA

Acidic Sphingolipids

Infusion 0.6 mL/h (MeOH)

Prec 290 scan [MCA 60 Scans (3s/scan)]

DP = -100 CE = -75 IS = -4500

Next category of compounds being analyzed by “Inside-out” sphingolipidomics:
Gangliosides (tentative assignments)

Raw cells +KdO2

Lipid A

Raw cells

Sulfatide

GM3 ➔ GM2 ➔ GM1 ➔ GD1a
Work-flow for analysis of new samples using this LC-MS/MS Methodology

1. Identify structure specific dissociations unique to various classes (e.g., SM, GlcCer, GalCer, LacCer, etc.)

2. Utilize precursor ion and neutral loss scans to identify individual headgroup, sphingoid base, and fatty acid combinations.

3. Optimize ionization and dissociation conditions for all species.

4. Optimize LC as required to minimize ionization suppression effects, and interferences arising from isobaric, isotopic, and isomeric species (repeat #3 if necessary).

5. Optimize conditions for quantitation via ratio of peak areas vs validated internal standards for all of the species present.
Example: Identification of sphingolipid subspecies via Neutral Loss or Precursor Ion Scans
D. Quantitation of Sphingolipids

Comparison of ion abundance for ceramides of varying chain length when analyzed under single ionization and dissociation conditions vs optimized MRM.
Criteria for selection of internal standards

1. Must have the same chemical and physical properties as the analyte of interest, ideally stable isotope labeled analogs.

2. Should be practical for “omic” analysis--i.e., cover as many subspecies as possible because adding an internal standard for every analyte would require 100s to 1000s of molecules to be synthesized, added and analyzed, which is too expensive, time consuming and possibly analytically impossible.

LIPID MAPS Sphingolipidomics cocktail (available from Avanti Polar Lipids): 10 uncommon sphingolipid species that are used to spike samples prior to extraction (Walt Shaw)
For sphingoid bases: odd chain length variants that elute under similar conditions so there is little ionization or dissociation effects and precursor and product ion masses are slightly shifted.

- d17:1 “sphingosine” and “sphingosine 1-phosphate” homologs
- d17:0 “sphinganine” and “sphinganine 1-phosphate” homologs

For complex sphingolipids: shorter fatty acid chain length variants (C12:0) that co-elute with analytes of interest so there are no ionization effects, and have different precursor ion masses but similar fragmentation when optimized.

- C12-Cer, C25-Cer, C12-Cer-1-P, C12-GlcCer, C12-LacCer, C12 SM

Also available: C12-Sulfatide
Under development: C12-GM1 and other complex glycosphingolipids
C17:1 sphingosylphosphochoiline; N-methyl-sphingoid bases
Analyzed by LC MS/MS using an ABI 4000 QTrap

Comparison of MRM for ceramides and dihydroceramides of varying chain length compared to C12-Cer internal standard
Example data from Lipid MAPS experiments:
Changes in Cer in RAW 267 cells treated with Kdo$_2$ Lipid A
(see www.lipidmaps.org)

Basic LIPID MAPS Protocol

Plate cells → Pre-treatments (if any) → Add agent (LPS, Kdo2 Lipid A) → Grow → Incubation → Analyze

E. Data analysis/visualization:
CORE I: Sphingolipids 
(only C16 shown)
Biological Replicates +/- SE

Data Sets Available at: www.lipidmaps.org 
(Eoin Fahy)
F. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies)--such as N-methylsphingoid bases

Obtain standards for glycosphingolipids (and new sphingolipids)

Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.

Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.
Example of Lipid MAPS timecourse data set for sphingolipids
(see www.lipidmaps.org)
Relational depiction of sphingolipid biosynthesis

How to display so much information?

Expand as additional species are analyzed

Key
- Sphingoid base (KSa, Sa, So, Etc.)
- N-acyl-sphingoid base (Fatty acid carbon #:db)
- Sphingoid base 1-phosphate
- N(Me2) sphingoid base
- Fatty acyl-CoA or fatty aldehyde (H’anal or enal)

-So -Glc
-So -Glc
-Phosphoryl/choline (Sphingomyelin)
-Phosphate (Ceramide 1-P)
F. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies).

Obtain standards for glycosphingolipids.

Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.

Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.
Analysis of sphingolipid biosynthesis using stable isotope labeled precursors

RAW 10% time course w/[\textsuperscript{13}C]palmitate

0.1 mM [\textsuperscript{13}C]palmitate
\[\downarrow\]
Ser + PalCoA
\[\downarrow\]
3-KetoSa
\[\downarrow\]
Sa
\[\downarrow\]
GlcDH Cer \[\rightarrow\] DH Cer \[\rightarrow\] DHSM
\[\downarrow\]
Glc Cer \[\rightarrow\] Cer \[\rightarrow\] SM

\textsuperscript{13}C BASE Ceramide (labeled sphingoid base only)

\textsuperscript{13}C DUAL Ceramide (\textsuperscript{13}C in sphingoid base and fatty acid)
To understand sphingolipid biosynthesis one must also know the availability of the co-substrate fatty acyl-CoA’s.

C16:0-CoA (10 pmol/µL in methanol 10 mM triethylammonium acetate) was infused at 5 µL/min. 4000QTrap parameters: IS = 5400 V, Gs1 = 12 psi, CUR = 10 psi, DP = 180 V, CE = 52 V, CXP = 14.3 V, CAD = Med
G. Examples of discoveries from sphingolipidomic analysis thus far

*de novo* sphingolipid biosynthesis is induced by Kdo2 Lipid A (has been correlated with gene array data showing increases in SPT1 and SPT2 mRNA)

Basic LIPID MAPS Protocol

- Plate cells
- Add agent (LPS, Kdo2 Lipid A)
- Grow
- Incubation
- Analyze

Examples of discoveries from sphingolipidomic analysis thus far:

- N-acyl chain subspecies
- LIPID MAPS
All subspecies have been quantified for these chain lengths, too (only a few are shown; all are at www.lipidmaps.org).

Sulfatide (3'-sulfogalactosylceramide)

Kdo2 Lipid A

Sulfatide <0.1 → 385 (exact quantitation still in progress)
G. Examples of additional discoveries from sphingolipidomic analysis thus far


H. Comparison of these methods with other sphingolipidomic techniques in the literature

"Shotgun" techniques: Use the same precursor ion / neutral loss scans - Great for profiling, not quantitation, suffer from ionization suppression, isotopic, isobaric, and isomeric interferences especially without hydrolysis and extraction.

Nanospray ionization: Greatly improved sensitivity and reduced chemical noise: allows detection of low abundance species, detailed structural analyses on numerous species, chip-based systems can be coupled to LC, and fraction collection.

Ultra high resolution mass analysis: allows differentiation of isobaric / isotopic interferences and alternative fragmentation techniques.