Analysis of novel *Caenorhabditis elegans*-specific phosphorylated glycosphingolipids using LC-TIMS-MS/MS and MassQL

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Introduction

The quest for new lipids

High-resolution mass spectrometry (HR-MS) is an important tool for lipid identification in different biological samples, with enhancements from orthogonal information such as retention times (RT) and collisional cross sections (CCS). The nematode *Caenorhabditis elegans* represents an important model organism in biomedical research, with many recent studies investigating intact lipids. As the entire *C. elegans* lipidome is not known yet, correct lipid annotation and identification are crucial. While for known lipid classes this can be achieved in a reproducible manner by rule-based annotation, the systematic search for novel lipid classes and species needs dedicated data analysis strategies. Here we present the analysis of phosphorylated glycosphingolipids (Fig 1) from *C. elegans* using Mass Spec Query Language (MassQL) to query for lipid characteristic MS/MS data.

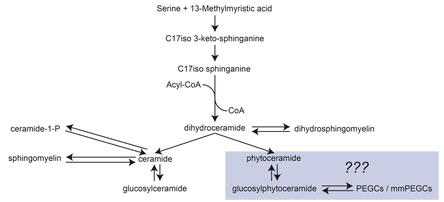

Fig. 1 Overview on *C. elegans* sphingolipid metabolism. The exact route for the biosynthesis of phosphoethanolamine glucosylceramides (PEGCs) and monomethyl phosphoethanolamine glucosylceramides (mmPEGCs) is not known, but most likely happens through phyto- and glucosylphytoceramides.

Boland *et al.* [1] recently described novel phosphorylated sphingolipids in *C. elegans*. Although the sphingolipidome of *C. elegans* has been thoroughly analyzed in previous studies [2, 3], this particular class of lipids was only detected during a cholesterol deprivation study. These sphingolipids are derivatives of glucosylceramides, characterized by an unusual phosphoethanolamine (PEGCs) or monomethyl phosphoethanolamine (mmPEGCs) residue linked to the sugar moiety (Fig 2A). Currently, there are no commercial reference standards for these lipids, and no reference MS/MS spectra have been deposited in public spectral repositories.

Methods

C. elegans reference samples were obtained from the University of Georgia (Athens, Georgia, US) and extracted with different lipid extraction protocols: Bligh and Dyer, MTBE, alkaline MTBE, BUME and MeOH. Analysis was performed using an Elute UHPLC coupled to a timsTOF Pro 2 (Bruker Daltonics). Lipid separation was achieved on a Waters Cortecs C18 column (150 mm x 2.1 mm ID; 1.6 µm particle size) with a gradient from 40% H_2O / 60% ACN to 10% ACN / 90% iPrOH, both with 10 mM ammonium formate and 0.1% formic acid. Data was acquired in positive and negative mode using DDA-PASEF and data analysis was performed in MetaboScape 2023b, which includes a beta-version of MassQL Based on the described fragmentation spectra, MassQL queries have been generated to search the obtained LC-TIMS-MS/MS data. An example query for mmPEGCs is shown in Fig 2B.

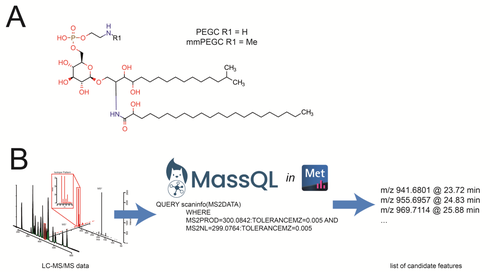

Fig. 2 (A) Example structures of PEGCs and mmPEGCs. Sphinoglipids in *C. elegans* are based on a C17iso sphingoid base. (B) Workflow for searching new lipids in LC-MS data. The use of MassQL generates a list of putative candidate features which can be further investigated, e.g. using RT and CCS trends.

Results

- MassQL allowed to identify candidates for novel species of phosphorylated glycosphingolipids (Fig 3A)
- Chromatographic separation and ion mobility enable additional filtering of false positive hits (Fig 3B and Fig 3C)
- Additional species for mmPEGCs have been identified
- Phytoceramides and phytohexosylceramides have been identified as potential biosynthetic precurcors (Fig 4)

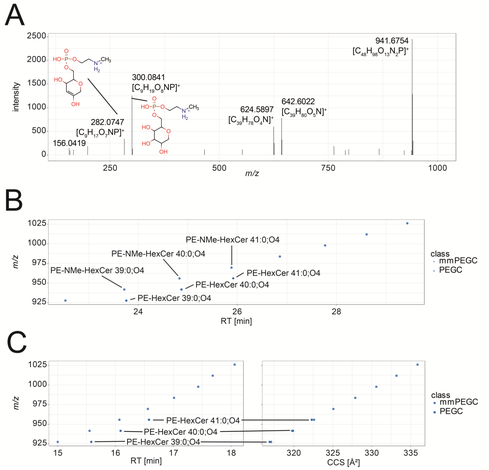

Fig. 3 (A) Example MS^2 of PE-NMe-HexCer 39:0;O4. (B) Chromatographic trendline of PEGCs and mmPEGCs identified in [2]. (C) Chromatographic and CCS trendlines from *C. elegans* reference samples. Isomeric PEGCs and mmPEGCs have very similar CCS values and only combination with chromatographic separation allowed the correct determination of CCS values. (see table below)

m/z	RT [min]	CCS [Å²]	Name	Detected by Boland *et al.*?
927.6644	15.58	316.9	PE-HexCer 39:0;O4	Y
941.6801	16.09	319.9	PE-HexCer 40:0;O4	N
955.6957	16.58	322.3	PE-HexCer 41:0;O4	Y
927.6644	15.00	317.1	PE-NMe-HexCer 38:0;O4	N
941.6801	15.55	319.8	PE-NMe-HexCer 39:0;O4	Y
955.6975	16.07	322.6	PE-NMe-HexCer 40:0;O4	Y
969.7114	16.56	325.1	PE-NMe-HexCer 41:0;O4	Y
983.7271	17.01	327.9	PE-NMe-HexCer 42:0;O4	N
997.7427	17.43	330.6	PE-NMe-HexCer 43:0;O4	N
1011.7583	17.68	333.2	PE-NMe-HexCer 44:0;O4	N
1025.7740	18.06	335.9	PE-NMe-HexCer 45:0;O4	N

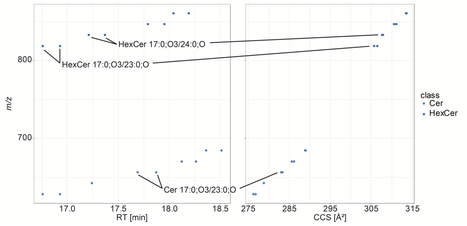

Fig. 4 Identified phytoceramides and phytohexosylceramides in *C. elegans* reference samples. Phytoceramides have been identified by rule-based lipid annotations, while candidate phytohexosylceramieds were annotated by MassQL and data refined using chromatographic and ion mobility separation.

Summary

For the first time, we report MassQL queries and CCS values associated with novel phosphorylated sphingolipids in *C. elegans*. Our approach led to the identification of six novel species with varying N-acyl chain lengths, in addition to five known species. To validate these MS/MS-based annotations, we examined trends along RT and CCS to study chromatographic and ion mobility behavior. This study marks the first time this lipid class has been reported with ion mobility. The obtained CCS values and fragmentation patterns can aid in the identification of these lipids in future studies.

Literature

[1] doi:10.1038/nchembio.2347

[2] https://doi.org/10.1016/j.chemphyslip.2019.04.009

[3] https://doi.org/10.1016/j.chroma.2021.462481.

Conclusion

- MassQL is a powerful tool for putative lipid annotation
- Combination of LC and TIMS in combination with MS/MS is important for structural annotation
- RT and CCS trendlines helped to reduce false positive annotations
- Several more species than originally described could be identified, including biosynthetic precursors of PEGC and mmPEGC

LC-TIMS-MS/MS