Electronic Supplementary Material (ESI) for Analytical Methods.


# SUPPLEMENTARY INFORMATION

# Checkpoints for Preliminary Identification of Small Molecules found Enriched in Autophagosomes and Activated Mast Cell Secretions Analyzed by Comparative UPLC/MS[e]

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***Sample Preparation***

*Enrichment of Autophagosomes from Rat Liver*

Male Wistar rats (200–250 g) were obtained from Jackson Laboratory (Fig. S1). Autophagosomes were isolated from rat liver after 6 h starvation using a protocol modified from the literature [1]. Livers were harvested, minced and homogenized using a Teflon homogenizer prior to separation by differential centrifugation. A pellet containing the nuclear fraction and (up to 30%) unbroken cells was produced first. The supernatant was centrifuged at 100,000xg and the pellet contained non-enriched organelles. Autophagosome fractions were enriched by differential centrifugation followed by discontinuous density metrizamide gradients as previously described.[1, 2]

*Animal Considerations.* All rat studies for autophagosome enrichment were conducted under an animal study protocol approved by the Albert Einstein College of Medicine Animal Institute Animal Care and Use Committee. Calorically restricted rats were allowed free access to water. All mice for mast cells were raised and euthanized according to an animal study protocol #0806A37663 approved by the University of Minnesota Institutional Animal Care and Use Committee.

*Enrichment for Autophagosomes from Rat Myoblast Cell Culture*

L6 rat myoblast cells, purchased from American Tissue Culture Collection (Manassas, VA) were cultured in 450 mL DMEM with 50 mL fetal bovine serum, and 100 µL gentamycin (Fig. S2). For the enrichment of autophagosomes, cells were first treated with vinblastine sulfate dissolved in 0.9% sodium chloride at a final concentration of 50 µM in DMEM solution for 2 hours. Vinblastine sulfate was used to increase the number of autophagosomes in the biological

system.[3, 4] Homogenization buffer consisted of 250 mM sucrose in deionized water (Millipore Synergy UV system, 18.2 mΩ/cm, Bedford, MA) adjusted to pH 7.2 with 100 mM sodium hydroxide. Cells were collected in homogenization buffer and centrifuged twice at 1,000g for 10 min. The cell pellet was resuspended in 1 mL homogenization buffer and 1% protease inhibitor cocktail. Cells were lysed with a Dounce homogenizer (clearance, 0.0025 in, Kontes Glass, Vineland, NJ). An organelle pellet was formed from the lysate with sequential 600g (to remove nuclei and cell debris) and 16,100g (to remove cytosol and cytosolic proteins) differential centrifugation steps. The resulting post-nuclear fraction was the control, non-enriched fraction. To enrich for autophagosomes, mitochondria were first immuno-removed with a Mitochondria Isolation Kit (MACS, Miltenyi Biotec, Auburn, CA), used according to the manufacturer's procedure and with the homogenization buffer described above. Following mitochondria removal, lysosomes were osmotically lysed with treatment of 500 nM glycyl-L-phenylnapthylamine for 8 min at 37 °C at 200 rpm on a thermomixer. Following treatment, the fraction was centrifuged for 30 min at 16,100g. Western blotting analysis revealed no detectable mitochondria and a highly reduced level of lysosome in the enriched autophagosome fraction (**Fig. S5**), consistent with previous published results for rat hepatocyte cells [5]. The resulting organelle pellet was defined as the enriched autophagosome fraction. Protein quantitation was done with the Pierce BCA protein assay kit (Thermo Fisher, Rockford, IL) according to the manufacturer's procedure.

*Extractions of Autophagosome Samples*

A previously published two step protocol for polar and nonpolar extractions was used for extractions of enriched autophagosome fractions from either rat liver or rat myoblast cell culture which resulted in the %CV of features from experimental replicates being < 30% from organic

fractions and being <20% from aqueous extracts [6]. Fractions were treated with 1.5 mL ice cold 1:1 v/v methanol:deionized water. Pellets were resuspended with a syringe, vortexed for 30 s, and incubated for 5 min. Samples were centrifuged at 16,100g for 10 min to pellet any non-extracted materials. The supernatant, defined as the polar extraction, was removed and kept on ice. The remaining pellet was treated with 1.5 mL ice cold 1:3 v/v dichloromethane:methanol. The pellet was resuspended with a syringe, vortexed for 30 s, and incubated for 5 min. The sample was centrifuged for 16,100g for 10 min to pellet any non-extracted materials. The supernatant, defined as the non-polar extraction, was removed and kept on ice. Extracted samples were vacuum concentrated overnight at room temperature to remove extraction solvent. Sample extracts were stored under nitrogen at -20° C until analysis.

*Safety Considerations*: All Biosafety Level 2 guidelines were followed when working with cell culture and tissue. Biological waste was bleached for 30 min prior to disposal.

*Stimulation of Mast Cells*

Murine peritoneal mast cells (MPMCs) were isolated via peritoneal lavage following euthanasia by $CO_2$ asphyxiation. Lavage was performed using approximately 8 mL per mouse of cell culture media composed of ice-cold high-glucose DMEM supplemented with 10% (v/v) BCS and 1% (v/v) PS, and 6-7 mL lavage fluid were recovered per mouse. After isolation, cells were pelleted at 450xg, resuspended in fresh media, and cultured overnight on a confluent monolayer of NIH/3t3 fibroblasts (purchased from American Type Culture Collection, Manassas, VA) with 0.5 µg/mL anti-TNP IgE.

MPMCs were washed three times with 37 °C Tris buffer (12.5 mM Trizma-HCl, 150 mM NaCl, 4.2 mM KCl, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 1.4 mM $MgCl_2$, pH 7.4) to remove cell

culture media, serum proteins, and cell debris. MPMCs were treated either with Tris buffer (2 cultures prepared) or with Tris buffer containing 200 ng/mL CXCL10 for 2 hr (2 cultures prepared) (Fig S3). Each pair of cultures was then treated with either Tris buffer or Tris buffer containing 100 ng/mL TNP-ova for 2 hr. These treatments in series created four different activation conditions: (1) Tris buffer followed by Tris buffer, (2) Tris buffer followed by TNP-ova, (3) CXCL10 followed by Tris buffer, and (4) CXCL10 followed by TNP-ova.

Supernatants were collected and filtered using PTFE 0.2 μm centrifugal filter units at 14,000g for 5 min, then stored overnight at -80 °C. Prior to UPLC/MS$^e$ analysis, samples were concentrated to 100 μL, and salts and proteins were precipitated via the addition of 1 mL ice cold ethanol. Samples were then centrifuged at 12,000xg for 10 min, and supernatants were vacuum concentrated to approximately 30 μL. Tris buffer/TNP-ova and CXCL10/TNP-ova samples were reconstituted to 300 μL with LC/MS-grade water and analyzed by UPLC/MS$^e$. Tris buffer/Tris buffer and CXCL10/Tris buffer samples were stored at -80 °C, then reconstituted to 300 μL with LC/MS-grade water prior to UPLC/MS$^e$ analysis. Unlike autophagosomes samples, mast cell secretions were not extracted into polar and non-polar fractions ahead of analysis.

***Biological Significance Mast Cell activation***

This analysis produced a variety of potential mast cell-secreted mediators. While more work is needed to confirm species identities, the initial results are sensible from a biological perspective. For example, 3-oxotetradecanoic acid (HMDB10730) is a known fatty acid involved in lipid synthesis and has a purported role in inflammatory response, and thus may be an interesting target to study in mouse models of various inflammatory conditions. In another example, D-pantetheine 4'-phosphate (CHEBI61723), a metabolite associated with coenzyme A

biosynthesis, has a role in fatty acid biosynthesis, as well as enzymatic synthesis of peptides and additional biologically active metabolites according to the ChEBI database [7]. D-pantetheine 4'-phosphate was enriched in mast cell samples exposed to CXCL10 and TNP-ova versus TNP-ova alone, suggesting that synergistic activation of mast cells triggers enzymatic pathways that are not triggered by the asthmatic pathway alone. These results may have implications for therapeutic treatment of inflammatory conditions. Benzylpenicilloic acid (CHEBI61220), another detected species, is a metabolite of the antibiotic penicillin, which was present in the cell culture conditions [8], suggesting that this antibiotic metabolite either produced intracellularly or was endocytosed by the mast cells during culturing, and subsequent secretion of this species took place upon mast cell activation and degranulation by TNP-ova. Overall, this suggests that the UPLC/MS$^e$ technique and workflow for data processing presented here are applicable to many different types of biological systems to determine preliminary identifications of small molecules specific to or enriched in a biological system.

**Table S1.** Number of Features and IDs at various steps of the workflow.

| System[1] | Ext[2] | ESI[3] | Data Features | | Selected Features Entering Checkpoint 1 | | | Candidate Features After Checkpoint 1 | | Database IDs | | Preliminary IDs After Checkpoint 2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Subtotal | | Subtotal #, (%)[5] | % CCF[4] | | Subtotal #, (%)[5] | | Subtotal #, (%)[5] | | Subtotal #, (%)[5] |
| Liver | Polar | + | 2086 | 3568 | 62 | 114 (3%) | 11 | 45 | 76 (67%) | 28 | 46 (61%) | 13 | 20 (46%) |
| Liver | NP | + | 1045 | | 49 | | 23 | 28 | | 18 | | 7 | |
| Liver | NP | - | 437 | | 3 | | 0 | 3 | | 0 | | 0 | |
| Myoblast | Polar | + | 1440 | 5072 | 56 | 130 (3%) | 19 | 51 | 112 (86%) | 37 | 69 (62%) | 4 | 8 (12%) |
| Myoblast | NP | + | 2008 | | 55 | | 22 | 45 | | 28 | | 3 | |
| Myoblast | NP | - | 1624 | | 19 | | 19 | 16 | | 4 | | 1 | |
| MCN (vs. MCCX) | NA | - | 4765 | 28622 | 10 | 2655 (9%) | 39 | 10 | 315 (12%)[6] | 1 | 114 (36%) | 0 | 15 (13%) |
| MCCX (vs. MCN) | NA | - | 4765 | | 11 | | 0 | 8 | | 6 | | 0 | |
| MCN (vs. MCTN) | NA | + | 1914 | | 398 | | 23 | 286 | | 3 | | 1 | |
| MCTN (vs. MCN) | NA | + | 1914 | | 181 | | 50 | 136 | | 24 | | 7 | |
| MCN (vs. MCTN) | NA | - | 2843 | | 487 | | 3 | 186 | | 12 | | 1 | |
| MCTN (vs. MCN) | NA | - | 2843 | | 266 | | 41 | 102 | | 23 | | 2 | |
| MCB (vs. MCCX) | NA | + | 1804 | | 187 | | 13 | 168 | | 19 | | 2 | |
| MCCX (vs. MCB) | NA | + | 1804 | | 449 | | 33 | 431 | | 2 | | 1 | |
| MCB (vs. MCCX) | NA | - | 2985 | | 248 | | 4 | 148 | | 20 | | 1 | |
| MCCX (vs. MCB) | NA | - | 2985 | | 418 | | 15 | 186 | | 4 | | 0 | |
| Totals | | | 37262 | 37262 | 2899 | 2899 (8%) | --- | 1849 | 503[6] | 229 | 229 (46%) | 49 | 43 (19%) |

[1]Liver = enriched autophagosomes from liver; myoblast = enriched autophagosomes from rat myoblasts; MCN = mast cells, non-activated; MCTN = TNP-ova-activated mast cells; MCCX = CXCL10-activated mast cells; MCB = Both CXCL10- and TNP-ova-activated.

[2]Extractions performed were either nonpolar (NP) or polar (see materials & methods). NA = no extraction made.

[3]ESI analysis was performed in positive (+) and negative (-) ionization modes.

[4] Overlap of common candidate features detected in a system by OPLS-DA and either LMM or t-test (% CCF).

[5]This column includes the total number (#) for each system regardless the nature of the extraction (NP, polar, or none). The value in parenthesis represents the fraction (%) of features or ideas relative to the previous step in the workflow described in Figure 1.

[6] Because of the large number of selected features from comparisons involving MCN, MCCX, MCTN and MCB, the top 250 candidate features of the LMM and the top 65 candidate features of the OPLS-DA analyses that passed Checkpoint 1 were searched in databases. Thus, 503 candidate features were searched in databases.

**Table S2.** Preliminary Identifications.

| Sample[1] | Extraction, ESI[2] | *m/z*[3] | Neutral Mass[3] | $T_R$[4] | Chemometrics/ statistics[5] | p-value[5] | FE[6] | Preliminary Identification[7] | Database[7] | ID Mass[8] | Mass Error (ppm)[8] | Mass Error (mDa)[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liver | NP + | 288.295 | 287.287 | 9.14 | t-test, OPLS-DA | $1.49 \times 10^{-5}$ | N.D. | HMDB00269 | LM, HM | 287.282 | 1.66 | 4.26 |
| Liver | NP + | 288.295 | 287.287 | 8.85 | t-test, OPLS-DA | $8.62 \times 10^{-6}$ | N.D. | HMDB00269 | LM, HM | 287.282 | 1.66 | 4.26 |
| Liver | NP + | 415.214 | 414.206 | 10.52 | t-test, OPLS-DA | $1.24 \times 10^{-3}$ | 1.85 | CHEBI31547 | CE | 414.204 | 0.51 | 1.60 |
| Liver | NP + | 623.451 | 622.443 | 17.76 | t-test, OPLS-DA | $1.09 \times 10^{-4}$ | 1.74 | LMGP01011228 | LM | 622.445 | 0.18 | -1.60 |
| Liver | NP + | 265.109 | 264.101 | 7.68 | t-test | $3.39 \times 10^{-4}$ | N.D. | CS124129 | CS | 264.100 | 0.57 | 1.00 |
| Liver | NP + | 244.268 | 243.260 | 8.89 | OPLS-DA | N.A. | 2.18 | CS59037 | CS | 243.256 | 1.93 | 4.20 |
| Liver | NP + | 244.268 | 243.261 | 9.22 | OPLS-DA | N.A. | 1.36 | CS8833239 | CS | 243.256 | 1.97 | 4.30 |
| Liver | P + | 361.276 | 360.268 | 15.10 | t-test | $9.73 \times 10^{-5}$ | N.D. | LMST03020020 | CE/LM | 360.266 | 0.61 | 1.70 |
| Liver | P + | 454.294 | 453.294 | 14.55 | t-test, OPLS-DA | $4.91 \times 10^{-6}$ | 11.6 | LMGP02050002 | HM/LM | 453.286 | 2.01 | 8.60 |
| Liver | P + | 522.355 | 521.347 | 14.98 | t-test | $1.81 \times 10^{-4}$ | N.D. | HMDB10385 | HM | 521.348 | 0.10 | -1.00 |
| Liver | P + | 524.371 | 523.362 | 15.99 | t-test, OPLS-DA | $2.58 \times 10^{-5}$ | 2.82 | LMGP01050026 | HM, LM | 523.364 | 0.19 | -1.50 |
| Liver | P + | 570.352 | 569.344 | 14.34 | t-test | $2.52 \times 10^{-6}$ | N.D. | HMDB10403 | CE, HM | 569.348 | 0.63 | -4.10 |
| Liver | P + | 300.294 | 299.286 | 13.11 | t-test | $4.23 \times 10^{-5}$ | N.D. | CHEBI16393 | All | 299.282 | 1.34 | 3.50 |
| Liver | P + | 367.143 | 366.135 | 0.54 | t-test | $3.19 \times 10^{-5}$ | N.D. | CHEMBL421556 | CS, CE | 366.136 | 0.11 | -0.90 |
| Liver | P + | 415.213 | 414.205 | 13.85 | t-test, OPLS-DA | $1.77 \times 10^{-4}$ | 3.23 | CS8129200 | CE/CS | 414.204 | 0.22 | 0.40 |
| Liver | P + | 415.213 | 414.205 | 13.41 | OPLS-DA | N.A. | 1.93 | CS9677319 | CS | 414.204 | 0.41 | 1.20 |
| Liver | P + | 482.324 | 481.316 | 15.90 | OPLS-DA | N.A. | 30.2 | HMDB11130 | HM | 481.317 | 0.00 | -0.50 |
| Liver | P + | 496.341 | 495.333 | 14.62 | OPLS-DA | N.A. | 2.60 | HMDB10382 | HM | 495.333 | 0.18 | 0.40 |
| Liver | P + | 520.339 | 519.331 | 13.99 | OPLS-DA | N.A. | 2.69 | HMDB10386 | HM | 519.333 | 0.12 | -1.10 |
| Liver | P + | 568.338 | 567.331 | 14.05 | OPLS-DA | N.A. | 9.22 | HMDB10404 | HM | 567.333 | 0.26 | -2.00 |
| Myoblast | NP - | 346.157 | 347.165 | 3.45 | LMM, OPLS-DA | $1.53 \times 10^{-18}$ | 79.3 | CHEBI51939 | CE | 347.163 | 0.35 | 1.70 |
| Myoblast | NP + | 286.273 | 285.265 | 9.88 | LMM | $3.78 \times 10^{-88}$ | N.D. | LMSP01040002 | LM | 285.267 | 0.35 | -1.50 |
| Myoblast | NP + | 288.291 | 287.283 | 9.26 | LMM, OPLS-DA | $6.80 \times 10^{-39}$ | N.D. | LMSP01040003 | LM | 287.282 | 0.35 | 0.50 |
| Myoblast | NP + | 545.312 | 544.305 | 16.76 | LMM | $1.44 \times 10^{-24}$ | 29.7 | CS8228689 | CS | 544.304 | 0.26 | 0.90 |
| Myoblast | P + | 273.168 | 272.160 | 9.11 | LMM | $6.65 \times 10^{-5}$ | N.D. | CS9087911 | CS | 272.164 | 1.10 | -3.50 |
| Myoblast | P + | 412.161 | 411.153 | 11.97 | LMM | $1.38 \times 10^{-20}$ | 59.4 | CS21513528 | CS | 411.158 | 1.07 | -4.90 |
| Myoblast | P + | 454.211 | 453.203 | 11.14 | LMM | $6.36 \times 10^{-6}$ | 44.7 | CS4644673 | CS | 453.205 | 0.35 | -2.10 |
| Myoblast | P + | 472.183 | 471.166 | 11.72 | LMM | $6.30 \times 10^{-12}$ | 40 | CS10484174 | CS | 471.166 | 0.11 | 0.00 |

**Table S2.** Preliminary Identifications (Continuation)

| Sample[1] | Extraction, ESI[2] | *m/z*[3] | Neutral Mass[3] | $T_R$[4] | Chemometrics/ statistics[5] | p-value[5] | FE[6] | Preliminary Identification[7] | Database[7] | ID Mass[8] | Mass Error (ppm)[8] | Mass Error (mDa)[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MCN (vs. MCTN) | NA- | 265.171 | 266.179 | 14.87 | OPLS-DA | N.A. | N.D. | CHEBI47781 | CE | 266.178 | 0.15 | 0.90 |
| MCTN (vs. MCN) | NA- | 241.180 | 242.188 | 14.52 | LMM | $7.06 \times 10^{-175}$ | 381 | HMDB10730 | All | 242.188 | 0.21 | 0.00 |
| MCTN (vs. MCN) | NA- | 447.132 | 448.140 | 12.66 | LMM | $4.24 \times 10^{-86}$ | 514 | CS4644613 | CS | 448.142 | 0.74 | -2.80 |
| MCN (vs. MCTN) | NA+ | 437.249 | 436.241 | 8.02 | LMM, OPLS-DA | $1.48 \times 10^{-3}$ | 1480 | CHEBI57835 | CE | 436.246 | 1.05 | -5.10 |
| MCTN (vs. MCN) | NA+ | 247.086 | 246.078 | 9.30 | LMM | 0 | 502 | CHEBI38130 | CE | 246.083 | 1.67 | -4.60 |
| MCTN (vs. MCN) | NA+ | 353.117 | 352.109 | 9.15 | LMM, OPLS-DA | 0 | 1190 | CHEBI61220 | CE | 352.109 | 0.03 | -0.60 |
| MCTN (vs. MCN) | NA+ | 355.064 | 354.056 | 9.24 | LMM, OPLS-DA | $1.49 \times 10^{-217}$ | 834 | CHEBI18337 | CE | 354.058 | 0.37 | -1.80 |
| MCTN (vs. MCN) | NA+ | 409.162 | 408.154 | 12.69 | LMM, OPLS-DA | 0 | 1220 | CS59230 | CS | 408.158 | 0.88 | -4.10 |
| MCTN (vs. MCN) | NA+ | 481.261 | 480.254 | 8.31 | LMM, OPLS-DA | 0 | 2200 | CHEBI2535 | CE | 480.262 | 1.73 | -8.80 |
| MCTN (vs. MCN) | NA+ | 525.289 | 524.281 | 8.47 | LMM, OPLS-DA | 0 | 1550 | CS20121381 | CS | 524.283 | 0.32 | -2.20 |
| MCTN (vs. MCN) | NA+ | 569.313 | 568.306 | 8.61 | LMM, OPLS-DA | 0 | 1010 | CS382892 | CE | 568.304 | 0.44 | 2.00 |
| MCB (vs. MCCX) | NA- | 265.146 | 266.154 | 15.12 | LMM, OPLS-DA | $1.06 \times 10^{-119}$ | 875 | CHEBI45599 | CE/CS | 266.155 | 0.34 | -1.40 |
| MCB (vs. MCCX) | NA+ | 525.291 | 524.283 | 8.36 | LMM, OPLS-DA | $1.53 \times 10^{-3}$ | 1530 | CS20121381 | CS | 524.283 | 0.02 | -0.60 |
| MCB (vs. MCCX) | NA+ | 234.207 | 233.199 | 8.90 | LMM, OPLS-DA | $4.44 \times 10^{-274}$ | 2200 | CS16127 | CS | 233.199 | 0.17 | -0.10 |
| MCB (vs. MCCX) | NA+ | 357.088 | 356.080 | 10.76 | LMM, OPLS-DA | $5.06 \times 10^{-282}$ | 1660 | CHEBI61723 | CE | 356.081 | 0.03 | -0.60 |

[1] Liver = enriched autophagosomes from liver; myoblast = enriched autophagosomes from rat myoblasts; MCN = mast cells, non-activated; MCTN = TNP-ova-activated mast cells; MCCX = CXCL10-activated mast cells; MCB = Both CXCL10- and TNP-ova-activated.

[2] Extractions performed were either nonpolar (NP) or polar (see materials & methods). NA = no extraction made. The "+" or "-" sign indicates that ESI analysis was performed in positive (+) and negative (-) ionization modes.

[3] Observed m/z values (***m/z* column**) was used to determine the neutral mass (**Neutral mass column**).

[4] $T_R$ is the observed retention time (min).

[5] Chemometrics/statistics that led to selection of a given feature were: OPLS-DA = Orthogonal partial least squares with discriminate analysis, LMM = Linear mixed model, or t-Test. The p-values correspond to significance determined with either LMM or t-test. N.A. = Not applicable.

[6] FE = Fold enrichment. N.D. = Not determined.

[7] Preliminary identifications were based on the following databases: CE = Chemical Entities of Biological Interest, HM = Human Metabolome Database, LP = Lipid Maps, and CS = Chemspider. "All" indicates that the compound was found in all four databases.

[8] Comparison of the ID Mass and the Neutral mass column was used to determine the error mass in mDa or ppm.

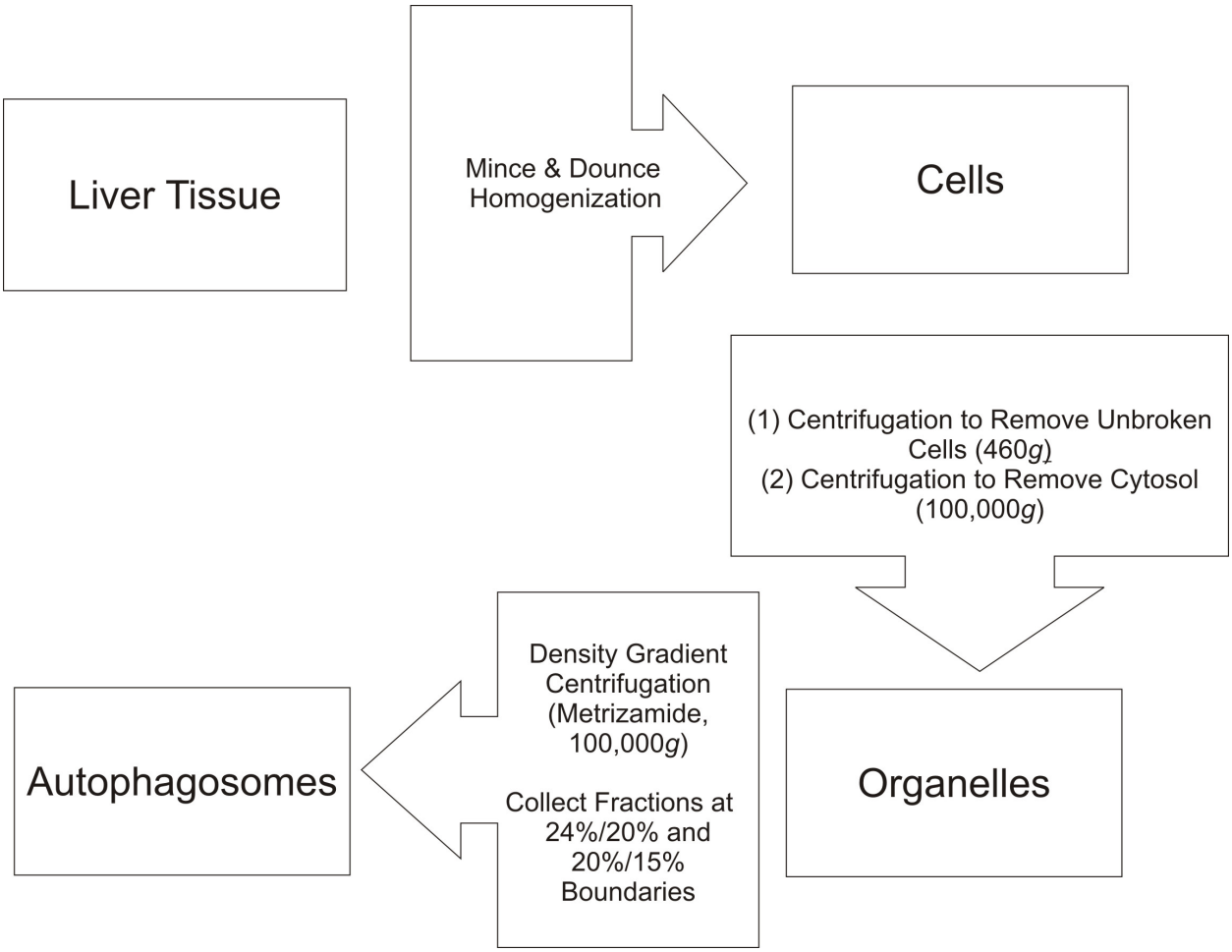


**Fig. S1** Flow diagram of enrichment of autophagosomes from rat liver tissue.

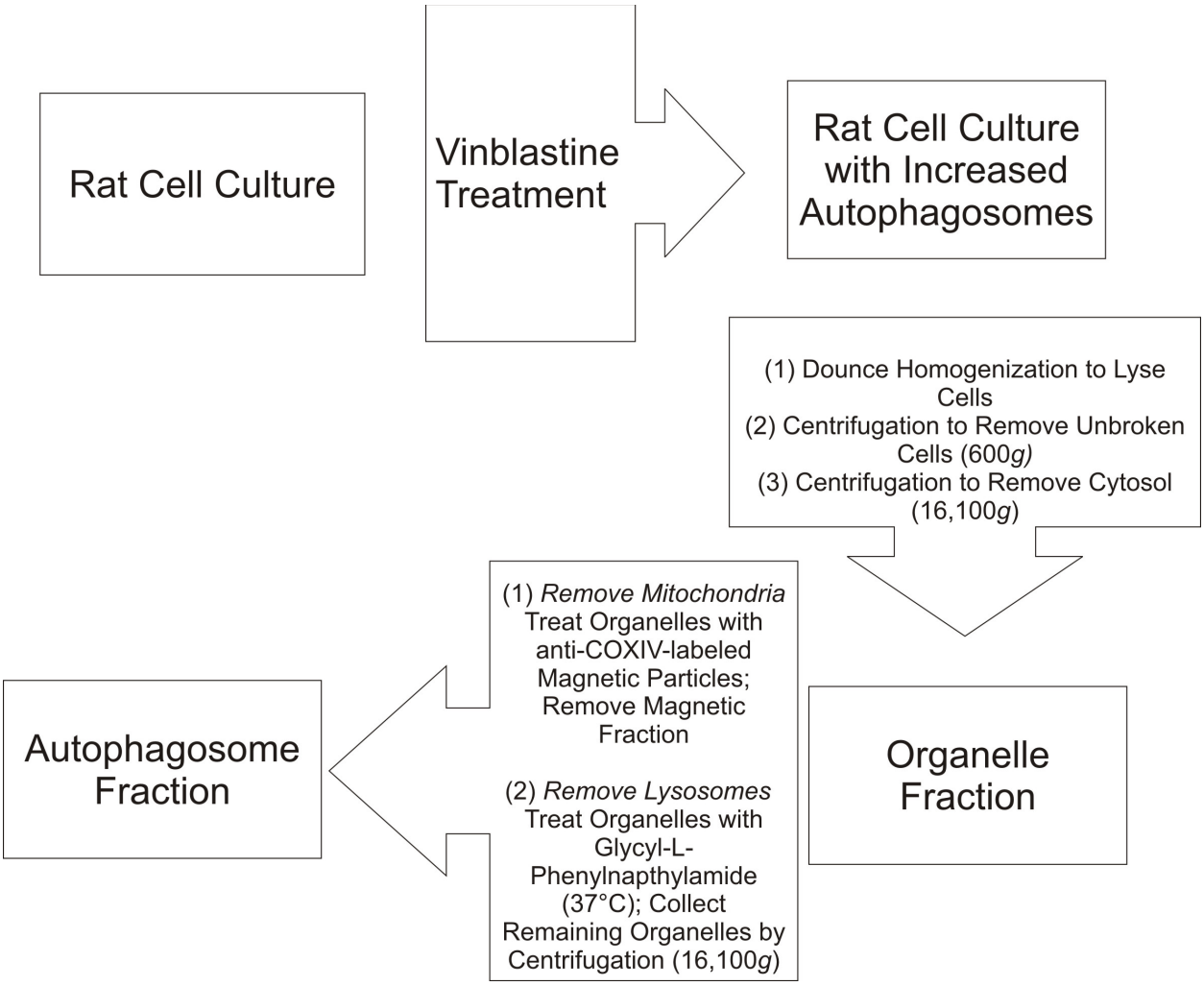


**Fig. S2** Flow diagram of enrichment of autophagosomes from rat myoblast cell culture.

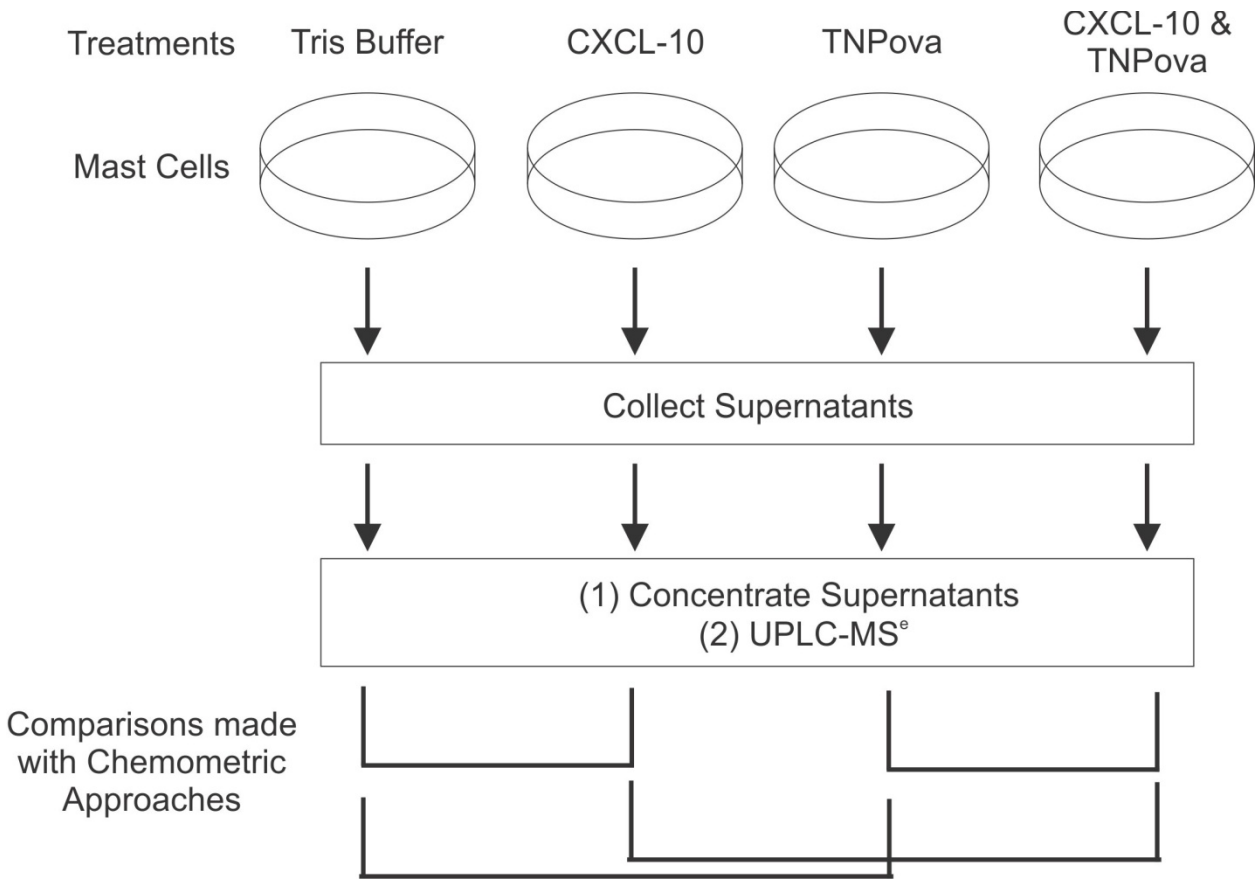


**Fig. S3** Flow diagram of mast cell treatments and comparisons.

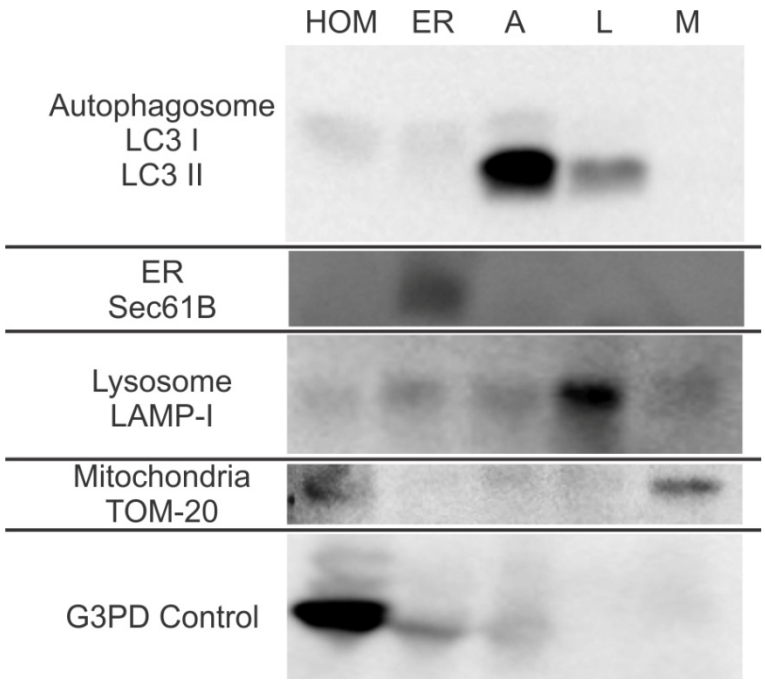


**Fig. S4** Western blots of rat liver autophagosome-enriched fractions revealed the autophagosome fraction was free of contamination from other organelles. From rat liver homogenate (HOM), a pellet containing the nuclear fraction and (up to 30%) unbroken cells was produced. The supernatant was centrifuged at 17000xg and the pellet is enriched in autophagosomes, lysosomes and mitochondria. The supernatant was centrifuged at 100000g and the pellet contains the vesicles in the non-autophagosome, endoplasmic reticulum enriched fraction (ER). From the 17000xg pellet fraction, autophagosome (A), lysosome (L), and mitochondria (M) were separated by differential centrifugation in discontinuous density metrizamide gradients as described. Experimental antibodies were anti-LC3 (1:500 v/v dilution, rabbit, NB-2220, Novus Biologicals, Littleton, CO), anti-SEC61B antibody (1:2000 v/v dilution, rabbit, NB100-74530) Novus Biologicals (Littleton, CO ), anti-LAMP1 antibody (1:1000 v/v, rabbit, ab24170, Abcam, Cambridge, MA), anti-Tom20 antibody FL-145 (1:500 v/v dilution, rabbit, sc-11415, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-G3PD antibody (1:500 v/v dilution, rabbit, 600-401-A33, 600-401-A33, Rockland, Gilbertsville, PA). Anti-rabbit (R21459) or anti-mouse (G21234) IgG (H+L) horseradish peroxidase secondary antibodies (1:5,000 v/v dilution, goat, Invitrogen, Carlsbad, CA) were used for chemiluminescence detection (Renaissance, NEN-Life Science Products). Membranes were exposed to BioMax Light Kodak films (Kodak Scientific Films) for increasing periods of time ranging from 5 s to 10 min.

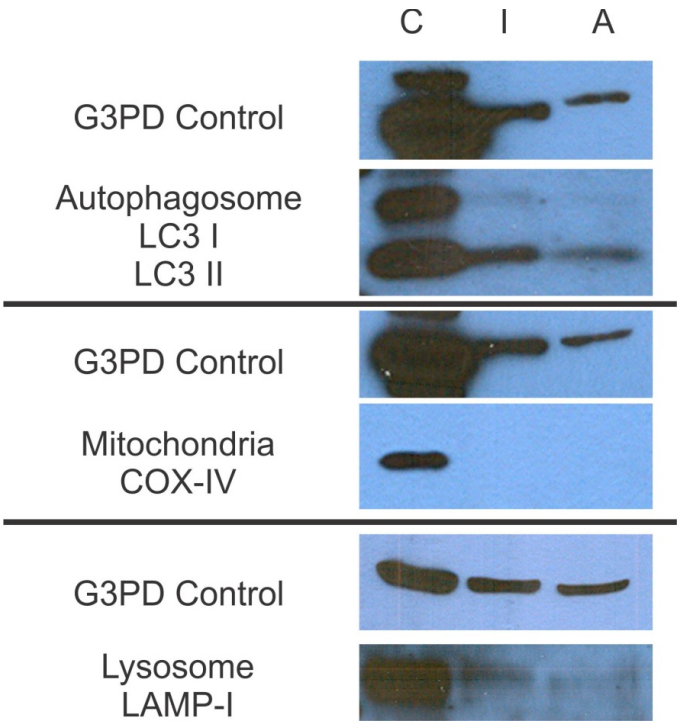


**Fig. S5** Western blots of autophagosome-enriched fractions from rat myoblasts show minimal mitochondria and lysosome contamination. Non-enriched, control organelle fraction (C), a semi-enrichment fraction (I), and the autophagosome-enriched fraction (A) were loaded onto a 15.0% "12+2" well SDS-PAGE gel (Criterion, Hercules, CA). Precision Plus Protein Dual Color standards (BioRad, USA) were used as a ladder for determination of molecular weights. The gel was run at 125 V for 60 min. Proteins were transferred onto a nitrocellulose membrane, 0.45 µm (BioRad, Germany). Transfer was done at constant voltage (100 V) for 2 h. Experimental antibodies were anti-LC3 (1:500 v/v dilution, rabbit, NB-2220, Novus Biologicals, Littleton, CO), anti-LAMP1 antibody (1:1000 v/v, rabbit, ab24170, Abcam, Cambridge, MA), and anti-COX-IV (1:500 v/v dilution, mouse, ab14744, Abcam,Cambridge, MA). Antibodies were diluted in 4% skim milk (Nestle, Eden Prarie, MN) in TBS/T solution (BioRad, USA). Anti-rabbit (R21459) or anti-mouse (G21234) IgG (H+L) horseradish peroxidase secondary antibodies (1:5,000 v/v dilution, goat, Invitrogen, Carlsbad, CA) were used for chemiluminescent detection. Membranes were imaged on a SRX-101A from Konica Minolta using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher, Rockford, IL). Glyceraldehyde 3-phosphate dehydrogenase (G3PD) was used as a positive control for the Western blotting system to ensure lanes contained protein. anti-G3PD antibodies were used for determination of G3PD as a loading control (1:500 v/v dilution, rabbit, 600-401-A33, Rockland, Gilbertsville, PA).

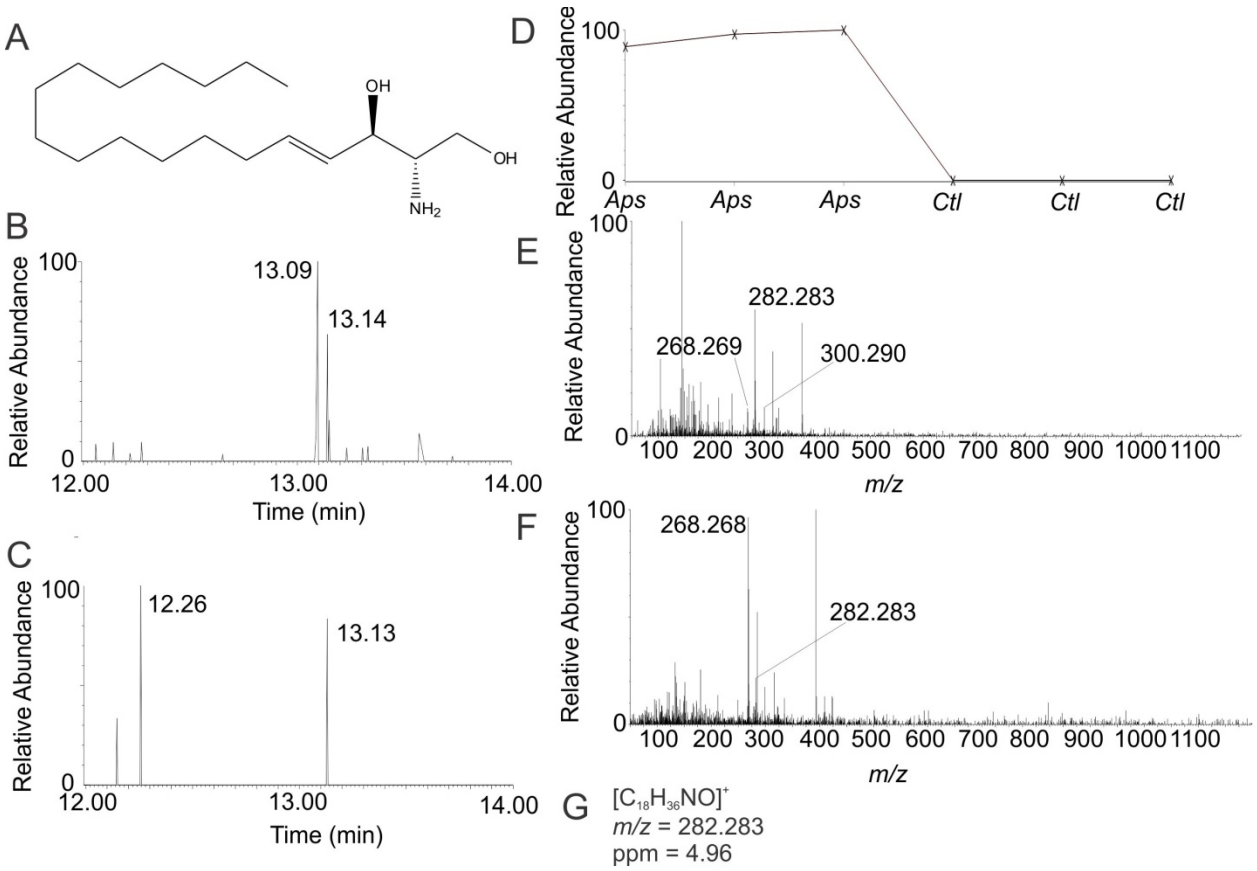


**Fig. S6** Preliminary identification of *m/z* 300.294 (2-aminooctadec-4-ene-1,3-diol) in autophagosomes-enriched fractions of rat liver ($T_R$ = 13.11 min, p-value = 4.23 x $10^{-5}$). (A) Structure of 2-aminooctadec-4-ene-1,3-diol; (B) Low-collision energy XIC for *m/z* = 300.294; (C) High-collision energy XIC for *m/z* = 300.294; (D) Trend plot for *m/z* = 300.294 in control (Ctl) versus autophagosome-enriched (Aps) samples; (E) Low-collision energy mass spectrum at $T_R$ = 13.11 min; (F) High-collision energy mass spectrum at $T_R$ = 13.11 min; (G) Elemental compositions, *m/z* values, and mass error of observed fragment ion corresponding to theoretical fragment ions generated *in silico* using Mass Fragment™.

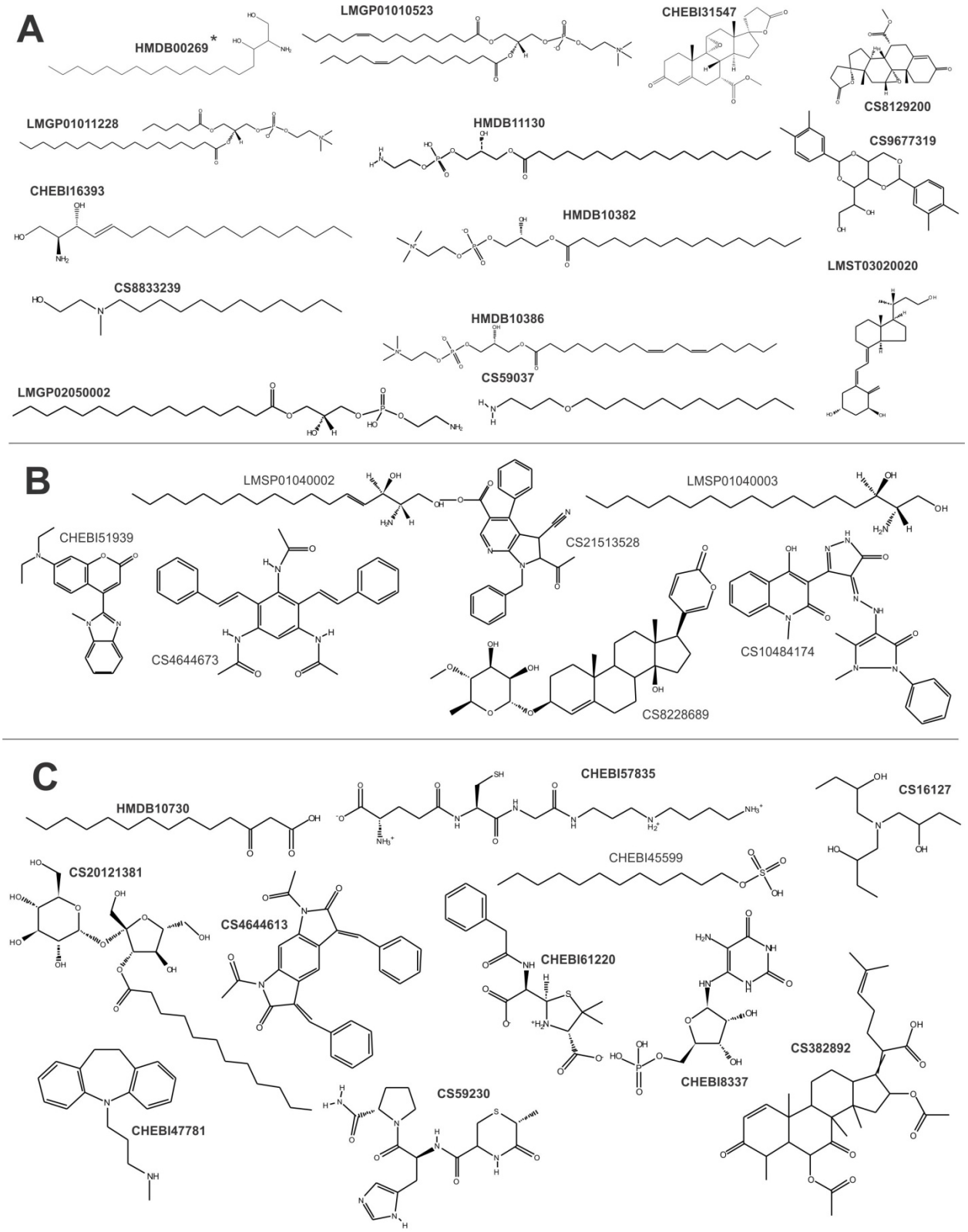


**Fig. S7** Examples of structures of preliminary identifications made for (A) rat liver autophagosomes, (B) rat myoblast autophagosomes, and (C) stimulant-activated mast cells. Two structural isomers of compound HMDB00269 (*) were detected in enriched rat liver autophagosomes (see Table 1).

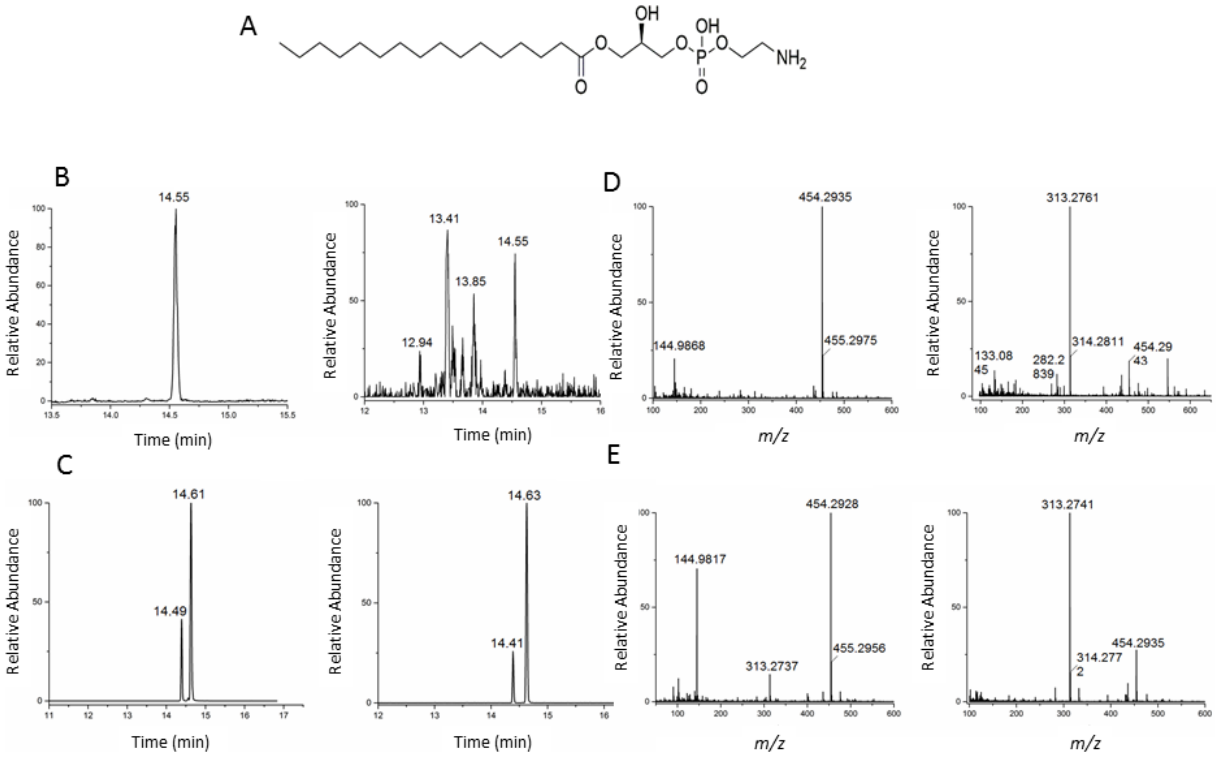


**Fig.S8** Confirming identification of *m/z* 453.2855 (1-hexadecanoyl-sn-glycero-3-phosphoethanolamine), ($T_R$ = 14.55 min). (A) Structure of 1-hexadecanoyl-sn-glycero-3-phosphoethanolamine; (B) Low and high-collision energy XIC for *m/z* = 453.2855; (C) Low and high-collision energy XIC for the standard *m/z* = 453.2855; (D) Low and high-collision energy mass spectrum at $T_R$ = 14.55 min; (E) Low and high-collision energy mass spectrum of the standard at $T_R$ = 14.55 min.

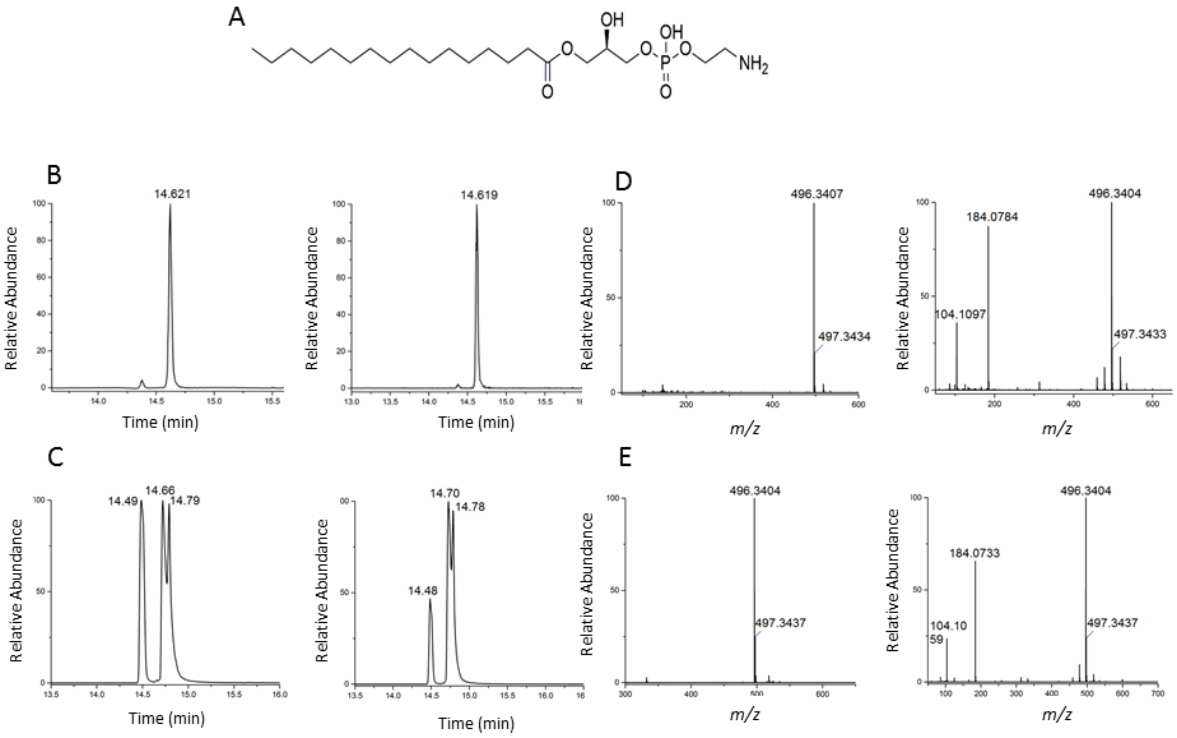


**Figure S9** Confirming identification of *m/z*= 495.3325 (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), ($T_R$ = 14.62 min). (A) Structure of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; (B) Low and high-collision energy XIC for *m/z* = 495.3325; (C) Low and high-collision energy XIC for the corresponding standard *m/z* = 495.3325; (D) Low and high-collision energy mass spectrum at $T_R$ = 14.62 min; (E) Low and high-collision energy mass spectrum of the standard at $T_R$ = 14.62 min.

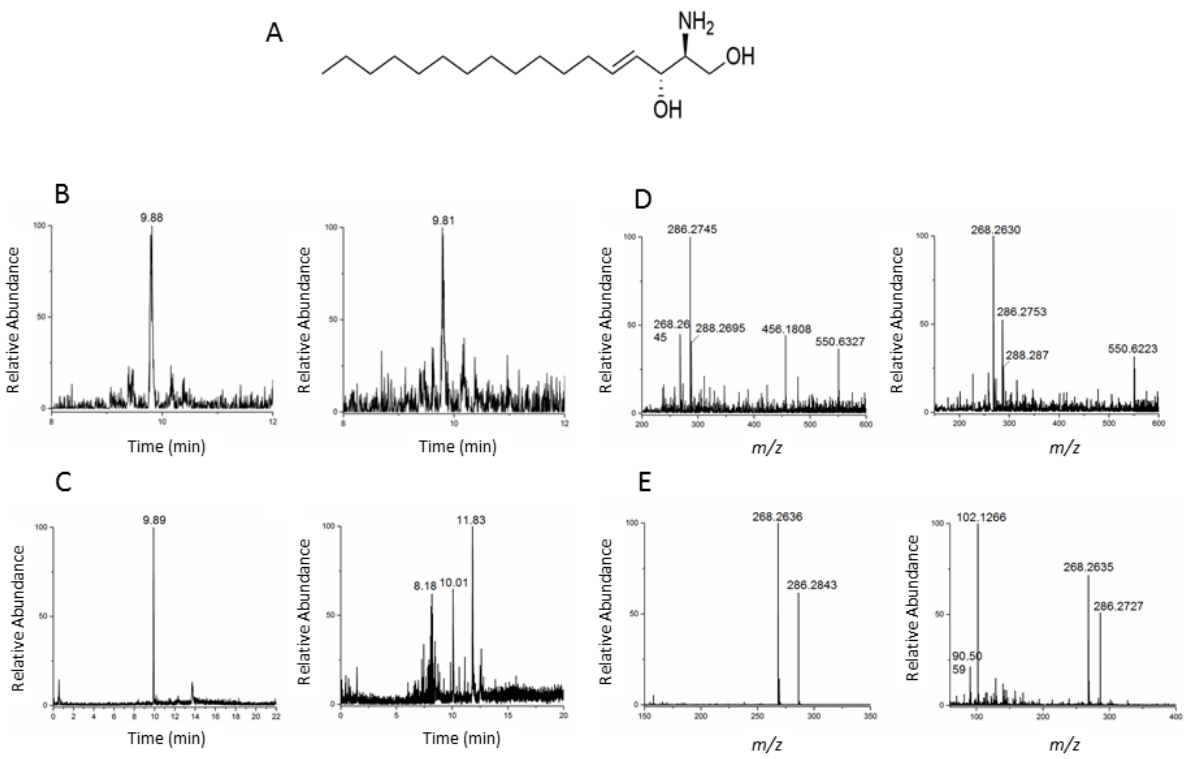


**Fig. S10** Confirming identification of *m/z* 285.2668 (D-erythro-sphingosine (C17 base), ($T_R$ = 9.88 min). (A) Structure of D-erythro-sphingosine (C17 base); (B) Low and high-collision energy XIC for *m/z* = 285.2668; (C) Low and high-collision energy XIC for the standard *m/z* = 285.2668; (D) Low and high-collision energy mass spectrum at $T_R$ = 9.88 min; (E) Low and high-collision energy mass spectrum of the standard at $T_R$ = 9.88 min.

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