Mapping of Lipid-Binding Proteins and Their Ligandability in Cells

Cravatt, B. F. et. al. Cell 2015, 161, 1668

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Literature Seminar
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Lipids can have structural (e.g. stabilizing membranes or proteins) or signaling roles (e.g. eicosanoids)

**Structural:**

Unusually positioned lipids hypothesized to influence structure and function of KcsA channel

**Signaling:**

![Diagram of lipid signaling pathways]

Arachidonic acid derived molecules mediate both physiological and pathophysiological signaling pathways

**References:**

Chemical Proteomic Probes to Characterize Lipid-Protein Interactions

Probe design based on small molecule-protein binding affinity and light-induced crosslinking to capture protein

**Design elements:**
- a) Small molecule to be recognized by protein ("lipid element")
- b) Photoreactive element that covalently links small molecule and protein upon UV irradiation
- c) Alkyne to allow late-stage conjugation to azide tag via Cu-catalyzed alkyne-azide cycloaddition

**Set of lipid probes:**

- **Diazirine photocrosslinking group**
- **Alkyne affinity handle**

- **Arachidonoyl (20:4) probes**
  - AEA-DA
  - AA-DA

- **Oleoyl (18:1) probes**
  - OEA-DA
  - O-DA

- **Palmitoyl (16:0) probes**
  - PEA-DA
  - S-DA

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Characterization of Lipid Probe Targets

Supplemental Figures

Cells

1. UV Light

Lipid Probe

Lipid probes

lipid

element

photoreactive

group

latex

affinity handle

Crosslinked

probe targets

1. CuAAC

rhodamine-azide

2. SDS-PAGE

Fluorescent

gel imaging

Identification of
target proteins

Rhodamine azide
fluorescent reporter tag

Rh–N₃

Cells incubated with probe for 30 min before UV

Cell 161, 1668–1680, June 18, 2015

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Lipid Probes Differentially Label Proteins

Lipid probes:

- **AEA-DA**
  - AA-DA
  - A-DA

**Figure 1.** Chemical Proteomic Probes for Mapping Lipid-Binding Proteins in Cells, Related to Supplemental Figures

- **Figure 1A:** Experimental workflow for gel-based profiling of lipid-binding proteins in mammalian cells. Cells were incubated with lipid probes for 30 min prior to crosslinking with UV light (10 min, 4°C) and subsequent cell lysis. Probe-labeled proteins were then conjugated to a rhodamine-azide (Rh-N₃) by copper-catalyzed azide-alkyne cycloaddition (CuAAC or "click") chemistry to allow for visualization of probe-labeled targets by SDS-PAGE and in-gel fluorescence scanning.

- **Figure 1B:** AA-DA and A-DA probes show overlapping protein-labeling profiles for the AEA-DA and AA-DA probes (20 μM, 30 min) in HEK293T cells. Red and blue arrows mark representative proteins preferentially labeled by AA-DA and A-DA probes, respectively.

- **Figure 1C:** Comparative protein labeling profiles of the A-DA, O-DA and S-DA probes in HEK293T cells, revealing a greater degree of labeling by the A-DA versus O-DA and S-DA probes.

- **Table:** Lipid Probes Differently Label Proteins
  - AEA-DA: labels almost exclusively membrane proteins
  - AA-DA: labels membranes, soluble proteins and phospho-proteins
  - A-DA: requires incubation and UV light to label proteins

- **Figure S1** demonstrates the distinct labeling profile of AEA-DA and A-DA probes, with AEA-DA showing preferential labeling of membrane proteins and A-DA showing dual labeling of membrane and soluble proteins. UV light irradiation is shown to enhance the labeling efficiency of these probes.

- **Conclusion:**
  - AA-DA probe labels almost exclusively membrane proteins
  - May be incorporated into phospho-neutral lipids or lipidedated proteins
  - Location of diazirine effects protein labeling profile
  - Protein labeling is UV dependent
  - Decide to use fatty acid amide probes
**Lipid Probes Differentially Label Proteins**

**Lipid probes:**

- **AEA-DA**
- **OEA-DA**
- **PEA-DA**
- **A-DA**
- **O-DA**
- **S-DA**

**Figure S1**

- (A) Structures of lipid probes featuring arachidonoyl (AEA-DA, AA-DA and A-DA), oleoyl (OEA-DA), and stearoyl (S-DA) acyl chains, as well as photoreactive (diazenylic) groups.

- (B) AEA-DA and A-DA probes show overlapping protein labeling profiles in HEK293T cells.

- (C) Comparative protein labeling profiles of the A-DA, O-DA and S-DA probes in HEK293T cells, revealing a greater degree of labeling by the A-DA versus O-DA and S-DA probes.

- (D) Comparative labeling profiles of lipid probes in HEK293T cells. Red and blue arrows mark representative proteins preferentially labeled by arachidonoyl and oleoyl/palmitoyl probes.

- (E) Preferential labeling by arachidonoyl probes.

- (F) Preferential labeling by oleoyl/palmitoyl probes.

- Polyunsaturated arachidonoyl probes (AEA-DA, A-DA) demonstrate more extensive protein labeling than monounsaturated (OEA-DA, O-DA) or saturated probes (PEA-DA, S-DA).

- Choose to map proteins that interact with arachidonoyl lipid probes.
Identification of Protein Targets with SILAC and LC-MS/MS

Stable-isotope labeling by amino acids in cell culture (SILAC) and LC-tandem MS (LC-MS/MS)

- Light cells are control and treated with arachidonoyl probe and UV
- Heavy cells are comparison
- Comparison heavy cell groups:
  a) Same conditions as light cells (probe-versus-probe control)
  b) Same probe as light cells but no UV (probe-versus-no UV)
  c) Other lipid probe (OEA-DA, O-DA, PEA-DA, S-DA)

- Lipid probe protein targets defined as proteins labeled in UV dependent manner (SILAC ratio ≥ 3.0 in probe-versus-no UV) and not enriched in probe vs. probe control (SILAC ratio < 2.0)
**Classification of Identified Protein Targets**

Identified protein targets include many with known links to lipid biology (e.g., enzymes and lipid carriers involved in fatty acid uptake, transport, biosynthesis, and catabolism) but also many without prior links.

*Figures:*
- **Diagram:** Highlighting lipid probe targets (red) in major fatty acid metabolic pathways.
- **Venn diagram:** Shared and unique protein targets of AEA-DA and A-DA probes in HEK293T and Neuro2a cells.
- **Heat map:** Relative enrichment values for lipid probe targets by the A-DA probe compared to O-DA and S-DA probes, as well as compared to the no UV control.

*Protein class distribution*:
- **Protein Classes**
  - Other
  - Enzymes
  - Transporters
  - Chaperones
  - Receptors
  - Adaptors
  - Channels

*Protein involvement in biological processes*:
- **Categories**
  - mRNA Processing
  - Apoptosis
  - Electron Transport
  - Host-Virus Interaction
  - Lipid Metabolism
  - Protein Transport

*Protein links to disease*:
- **Categories**
  - Hematological
  - Renal
  - Developmental
  - Reproduction
  - Immune
  - Psychiatric
  - Neurological
  - Cardiovascular
  - Cancer
  - Metabolic

*Known or predicted subcellular distribution*:
- **Proteins**
  - Cytosol
  - Nucleus
  - ER
  - Cell Membrane
  - Golgi
  - Lysosome
**Lipid-Interaction Proteome Enriched in Known Drug Targets**

- 25% of the identified lipid interaction proteome enriched in drug targets, while 12% of total human proteome is drugged
- Suggests lipid probes may preferentially interact with proteins that can bind other small molecule ligands
- Hypothesize that lipid probes can provide methods to determine drug target engagement and selectivity

Prostaglandin biosynthetic enzymes PTGS1 and PTGS2 are lipid probe targets:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Neuro2a cells</th>
<th>A549 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTGS1</td>
<td>Light Heavy A-DA (NoUV) A-DA</td>
<td>Light Heavy A-DA (NoUV) A-DA</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Light Heavy A-DA (NoUV) A-DA</td>
<td>Light Heavy A-DA (NoUV) A-DA</td>
</tr>
</tbody>
</table>

Inhibitors for PTGS1 and PTGS2 are known:

- **(±)-Flurbiprofen**
  - *PTGS1/2 inhibitor*
  
- **Rofecoxib**
  - *PTGS2 inhibitor*
Competition Between Lipid Probe and Drug For Protein Engagement

**Competition experiment:**

1. **DMSO** (Light Cells) and **Competitor** (Heavy Cells)
2. i) UV Light
   ii) Cell Lysis
3. Competed target
4. Mix
   - "Click" Enrich Digest
5. LC-MS/MS Analysis
   - Non-competed target
   - Competed target
6. Protein ID and quantification

**Competitors:**
- *(±)-Flurbiprofen*  
  *PTGS1/2 inhibitor*
- **Rofecoxib**  
  *PTGS2 inhibitor*

**MS1 intensity:**
- **SILAC Ratio**
  - Light
  - Heavy

**Graphs:**
- **Light Cells** vs. **Heavy Cells**
- **PTGS1 (Neuro2a)**
- **PTGS2 (A549)**

**Competition consistent with known inhibitor selectivity**

- PTGS enzymes are among the most competed A-DA target proteins, indicating good selectivity
- AKR1B8 is mouse ortholog of human aldo-keto reductase which is modified/ inhibited by prostaglandins
Lipid Probes as Screening Tool for Discovery of New Ligands

- Nucleobindin protein NUCB1 known to interact with PTGS1 and PTGS2 and enhance PTGS2-mediated prostaglandin synthesis, but not before known to bind small molecule ligands
  - Hypothesized to play role in cellular lipid metabolism
MJN228 Competes Arachidonoyl Probe for NUCB1 Binding

**NUC1B ligands:**

MJN228 (R₁ = Ph; R₂ = H)
KML110 (R₁ = Ph; R₂ = Me)
KML181 (R₁ = H; R₂ = H), inactive control

**Inhibition of AEA-DA probe labeling of NUCB1 in cells by new ligands**

**Competitive binding of optimized ligands to purified NUCB1 relative to FI-AEA probe**

**Table showing IC₅₀ values:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (95% CI), μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJN228</td>
<td>3.3 (2.9–3.9)</td>
</tr>
<tr>
<td>KML110</td>
<td>9.6 (7.8–11.9)</td>
</tr>
<tr>
<td>KML181</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

**Figure 7. Target Engagement and Lipid Metabolism Effects of NUCB1 Ligands**
- (A) Structure of Fl-AEA probe.
- (B) SILAC ratio plot for in situ competition experiment performed with MJN228 (10 μM).
- (C) Neuro2a Cells - AEA-DA (5.0 μM) vs. Competitor (25 μM).
- (D) Untargeted metabolite profiling reveals that Neuro2a cells treated with MJN228 (10 μM) show elevated fatty acid amides (NAEs and NATs) compared to cells treated with DMSO or KML181 (10 μM), but not KML181, FK-866 (25 μM), or avasimibe. See also Figures S6B-S6D for summary of medicinal chemistry.
- (E) Targeted MRM measurements showing elevations in NAEs in Neuro2a cells treated with NUCB1 ligands MJN228 and KML110 (10 μM) (p < 0.0001, n = 5 per condition). See also Figures 6G and 6H).
- (F) Left, western blot showing knockdown of NUCB1 in shNUCB1 A549 cell lines compared to a control cell line (shGFP). Right, both shNUCB1 cells show inactivates AEA-DA binding to NUCB1 over ~400 other probe targets.

**Additional content:**
- Deeper profiling of Neuro2a cells using the isoTOP-ABPP platform identified 150 proteins (Table S4), which accounted for ~400 other probe targets.
- MJN228 sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. Importantly, the MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. The MJN228-sensitive, AEA-DA-modified peptide resides within the previously mapped PTGS1/2-binding domain of NUCB1. Inhibitory effect of MJN228 inhibits AEA-DA probe binding to NUCB1 over ~400 other probe targets.
**Metabolic Effects of NUCB1-ligand Interaction**

**NUC1B ligands:**

MJN228 (R₁ = Ph; R₂ = H)  
KML110 (R₁ = Ph; R₂ = Me)  
KML181 (R₁ = H; R₂ = H), inactive control

**Identifying metabolic consequences of NUCB1-MJN228 interaction:**

Cell treatment with MJN228 leads to elevated levels of N-acyl ethanolamines (NAEs) and N-acyl taurines (NATs), two classes of fatty acid amides.

NAEs and NATs are both metabolized by the enzyme fatty acid amide hydrolase (FAAH), but neither MJN228 or KML110 inhibit FAAH (PF-7845 is known FAAH inhibitor).

**Figure S7**

- Site on NUCB1 of both ADA-DA probe and ligand MJN228 binding mapped to PTGS1/2 binding domain
- Suggests common region for NUC1B lipid-protein and protein-protein interactions

**Figure 7**

- AEA-DLabeled Peptide (aa 53-68)  
  [R.YLQEVINVLETDGH⁺FR.E]⁺⁴

- Light: AEA-DA + DMSO  
- Heavy: AEA-DA + MJN228 (1x)

- SILAC Ratio (L/H) = 5.4

**Table S4**

<table>
<thead>
<tr>
<th>Metabolite Changes with:</th>
<th>MJN228</th>
<th>MJN228 &amp; KML181</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 596</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z 622</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z 326</td>
<td>NAE (C18:1)</td>
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<tr>
<td>m/z 328</td>
<td>NAE (C18:0)</td>
<td></td>
</tr>
<tr>
<td>m/z 446</td>
<td>NAT (C24:0)</td>
<td></td>
</tr>
<tr>
<td>m/z 474</td>
<td>NAT (C26:0)</td>
<td></td>
</tr>
<tr>
<td>m/z 444</td>
<td>NAT (C24:1)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7**

- Recombinant hFAAH (AEA Substrate)  
  - 50 μM  
  - 100 μM

- DMSO  
- MJN228  
- KML110  
- KML181  
- PF-7845 (1.0 μM)
Metabolic Effects of NUCB1-ligand Interaction

NUCB1 ligands:

MJN228 (R₁ = Ph; R₂ = H)
KML110 (R₁ = Ph; R₂ = Me)
KML181 (R₁ = H; R₂ = H), inactive control

Knocking down expression of NUCB1 leads to elevated levels of fatty acid amides

Data collectively suggests that NUCB1 plays indirect role in facilitating fatty acid amide metabolism, e.g. serving as intracellular carrier to deliver lipids to fatty acid amide hydrolase (FAAH)
Globally Mapping Lipid-Binding Proteins and Their Ligandability

Proteomic probes allow mapping of cellular targets of small molecules and discovery of new small molecule protein ligands.