Small Molecule Modulators of Lipid Production in Microalgae and NMR Spectroscopy of Lipids

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Progress in Algae Research, Metabolic Regulation
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Algae biofuel research in the Franz Group

Increasing Lipid Production

Understanding Lipid Metabolism

Lipid Analysis

Biofuel Conversion

Fluorescence

Anderson et al., unpublished

C_{F} = 100 \frac{I_{ME}}{I_{ME} + 9 \cdot I_{TG}}


Microalgae are Photosynthetic Factories

Chisti, Y. Biotechnology Advances 2007, 25, 294
Wijffels, R. H.; Barbosa, M. J. Science 2010, 329, 796.
Methods and Barriers for Modulating and Understanding Microalgae Lipid Metabolism

- Nutrient limitation or “stress”
- Heterotrophic growth conditions
- Genetic manipulation
  - Difficult but advancing
  - Algae model species
    - *Chlamydomonas reinhardtii* (“the green yeast”)
    - *Phaeodactylum tricornutum*

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**Methods and Barriers for Modulating and Understanding Microalgae Lipid Metabolism**

<table>
<thead>
<tr>
<th>Day</th>
<th>Normal</th>
<th>Nitrogen Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Lipid Analysis of *P. tricornutum* by LSCM Imaging**

**Growth Analysis of *P. tricornutum***


Subcellular localization and multigene stacking: Rasala and Mayfield et al., PLoSONE, 2014


Radakovits et al., Euk Cell, 2010
Chemical Genetic Approach to Modulate Algae Lipid Production

Classical Genetics

Mutant Gene

Features of Chemical Genetics
1) Direct changes in real-time
2) Phenotype can be reversible
3) Concentration effects
4) Temporal effects
5) Synergistic compound effects
6) Combine for use with engineered strains

Chemical Genetics

Small Molecule

Protein

Phenotype

Wild-Type

“Mutant”

Schreiber, 1998, Bioorg Med Chem
Organic Molecules for Dynamic Information Flow

- DNA
  - replication
  - transcription
- RNA
  - translation
- proteins

Small molecules: signaling, communication, probes, medicines

Small molecules (signaling, probes, medicines)
Screening Approach

Phase I: microplate screening & Phase II: dose response screening

1. Add algae and media
2. Grow in microplates
3. Add chemical trigger
4. Add Nile Red (lipophilic dye)
5. Measure lipid production
6. Select chemical triggers

Phase III: 10+ selected lead compounds for 500 mL cultures

1. Add chemical trigger
2. Grow in batch cultures
3. Harvest algae
4. Extract lipids
5. Analyze lipids by MS, NMR, and microscopy
6. Increase understanding of algae lipids and metabolism

Initial Screening Set

- Kinase signaling
  - BPDQ
  - cAMP
  - Forskolin
  - CDK212
  - AICAR

- Lipid signaling
  - Orlistat
  - Quinacrine

- Protein synthesis
  - Cycloheximide

- Bioactivity
  - Antioxidant
  - Oxidative signaling
  - Cell cycle regulation
  - Plant growth
  - Anti-carcinogenic
  - Anti-proliferative
  - Anti-inflammatory
  - Anti-fungal

- Chemical Structure
  - Phenolics
  - Flavones
  - Terpenoids
  - Purine derivatives
  - Alkaloids
  - Triazoles
  - Oxindoles
  - Fatty acids
  - Indole-derived maleimides

- Other (e.g. phytohormones)
Diverse Compound Screening Reveals Small Molecule Modulators

- N. salina
- N. oculata
- Nannochloris sp.
- P. tricornutum

Analysis performed with Spotfire
Diverse Compound Screening Reveals Small Molecule Modulators

Compounds such as:
- epigallocatechin gallate
- PTP Inhibitor II
- CDK2 Inhibitor 2
- MAP sb202190
- quercetin
- kinetin
- cycloheximide

Legend:
- Growth Increase
- Lipids

Analysis performed with Spotfire
Chemical Triggers in 500 mL Cultures

<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Specific Growth Rate (day(^{-1}))</th>
<th>Lipid Content (% w/w)</th>
<th>Lipid Productivity (mg L(^{-1}) day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23 ± 0.06</td>
<td>23.6 ± 9.0</td>
<td>4.7 ± 1.4</td>
</tr>
<tr>
<td>Quinacrine (40 nM)</td>
<td>0.25 ± 0.02</td>
<td>26.5 ± 3.9</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>EGCG (40 μM)</td>
<td>0.22 ± 0.06</td>
<td>23.1 ± 10.6</td>
<td>6.3 ± 2.2</td>
</tr>
<tr>
<td>DMSO (0.4%)</td>
<td>0.26 ± 0.07</td>
<td>24.3 ± 7.5</td>
<td>6.5 ± 1.7</td>
</tr>
<tr>
<td>cAMP (4 μM)</td>
<td>0.22 ± 0.03</td>
<td>28.6 ± 10.7</td>
<td>7.9 ± 3.4</td>
</tr>
<tr>
<td>EGCG (in water) (4 μM)</td>
<td>0.24 ± 0.01</td>
<td>32.4 ± 0.1</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>BHA (4 nM)</td>
<td>0.23 ± 0.00</td>
<td>28.8 ± 11.4</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>Propyl gallate (40 nM)</td>
<td>0.23 ± 0.00</td>
<td>25.9 ± 11.4</td>
<td>8.3 ± 1.7</td>
</tr>
</tbody>
</table>

**N. salina**

**Oxidative signaling**

- Control
- Nitrogen Deficient (33%)
- DMSO (0.4%)
- EGCG (40 μM)

Propyl gallate

BHA

Epigallocatechin gallate (EGCG)
### Cost Analysis for Lead Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Price/gram</th>
<th>Concentration (identified in screening)</th>
<th>Amount needed</th>
<th>Price per dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>$1,200.00</td>
<td>4 mM</td>
<td>91.68 g</td>
<td>$110,016.00</td>
</tr>
<tr>
<td>cAMP</td>
<td>$107.00</td>
<td>4 mM</td>
<td>65.84 g</td>
<td>$7,044.88</td>
</tr>
<tr>
<td>Forskolin</td>
<td>$4,950.00</td>
<td>4 nM</td>
<td>0.0821 g</td>
<td>$406.40</td>
</tr>
<tr>
<td>BHA</td>
<td>$0.06</td>
<td>4 nM</td>
<td>0.036 g</td>
<td>$0.002</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>$0.09</td>
<td>40 nM</td>
<td>0.4244 g</td>
<td>$0.04</td>
</tr>
<tr>
<td>DMSO</td>
<td>$64.16/L</td>
<td>0.04%</td>
<td>20 L</td>
<td>$1,283.20</td>
</tr>
</tbody>
</table>

*crude sources of chemical triggers*
What pathway are our small molecules acting through?

Pathways and targets:
- Antioxidant and oxidative stress
- Signaling kinases
- Lipid metabolism
- Fatty acid oxidation
- Nutrient transport
- Growth stimulation and cell division
What metabolomics can tell us

What *can* happen

Genome

DNA

What *appears* to be happening

Transcriptome

RNA

What *makes* it happen

Proteome

Proteins

What *has* happened and *is* happening

Metabolome

Metabolites
- metabolic intermediates
- hormones and signaling molecules
- secondary metabolites (e.g. pigments)

Sugars
Nucleotides
Amino acids
Lipids
Metabolomics Work Flow

Sample collection and preparation

“quenching”

Data acquisition

Database curation and statistical analysis

Following biochemical pathways and networks

Bioinformatics

Modeling metabolic interactions

- Mechanistic insight
- Toxicology
- Classification
- Prediction
- Functional genomics


Collaboration with Fiehn Lab
Pattern of regulated features was detected according to nitrogen deficiency.
Lipidomics of *P. tricornutum* under nitrogen deficiency displays distinct differences

**On going experiments:** treatment with antioxidants such as EGCG will increase metabolites and lipids relating to antioxidant defense system, membrane integrity, and osmoprotection, along with changes in TCA cycle intermediates that correlate to fatty acid biosynthesis.

Anderson et al., unpublished

Collaboration with Fiehn Lab
Lipid Composition by $^1$H NMR Spectroscopy

Representative TAG

<table>
<thead>
<tr>
<th></th>
<th>SFAs (%)</th>
<th>UFAs (%)</th>
<th>w3 PUFAs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive Oil</td>
<td>15</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>Peanut Oil</td>
<td>14</td>
<td>86</td>
<td>2</td>
</tr>
<tr>
<td>N. salina</td>
<td>31</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td>N. oculata</td>
<td>24</td>
<td>76</td>
<td>28</td>
</tr>
<tr>
<td>T. suecica</td>
<td>21</td>
<td>79</td>
<td>16</td>
</tr>
<tr>
<td>T. suecica (glycerol)</td>
<td>44</td>
<td>56</td>
<td>9</td>
</tr>
<tr>
<td>P. tricornutum</td>
<td>21</td>
<td>68</td>
<td>11</td>
</tr>
</tbody>
</table>

Anderson et al., unpublished
Conclusions

Increasing Lipid Production

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C_{F} = 100 \frac{I_{ME}}{I_{ME} + 9 \cdot I_{TG}}


Anderson et al., unpublished


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Nitrogen limitation increases TCA cycle intermediates

Dong, H. P., et al., *Plant physiol* 2013, 162, 1110.
Dose-Dependent Activity

Microplate screening in water
Small molecule modulation of lipid metabolism in other organisms

**Triazine library for improved biofuel generation in yeast**

![Chemical structure of triazine library with labels for different functional groups and a graph showing fluorescence intensity.](image)


**Screening for small-molecule modulators of lipid storage in *C. elegans* for disease applications**

![Graph showing Nile Red fluorescence intensity for different treatments.](image)


**Triggering lipid droplet fusion with small molecules for disease applications**

![Diagram showing lipid droplet fusion with different treatments.](image)

Murphy et al., *PLoS ONE* 2010, 5, e15030.
Beyond Biofuels

Chemical Screen to Identify GPCRs as Regulators of Cilia in *C. reinhardtii*


Microalgal Bioremediation and Pollution Monitoring


*C. reinhardtii* mutants for anticancer drug screening


Phytohormone signaling, photosynthesis and protection