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Phenotypic screening identifies Brefeldin A/Ascotoxin as an inducer of lipid storage in the algae *Chlamydomonas reinhardtii*

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ABSTRACT

The use of microalgae as a biofuel feedstock is highly desired, but current methods to induce lipid accumulation cause severe stress responses that limit biomass and, thus oil yield. To address these issues, a high throughput screening (HTS) method was devised to identify chemical inducers of growth and lipid accumulation. Optimization was performed to determine the most effective cell density, DMSO and Nile Red (NR) concentrations to monitor growth and lipid accumulation. The method was tested using 1717 compounds from National Cancer Institute (NCI) Diversity Set III and Natural Products Set II in *Chlamydomonas reinhardtii*. Cells were inoculated at low density and 10 μM of the test compound was added. After 72 h, cell density was measured at OD_{550} and lipid accumulation assessed using NR fluorescence. Primary screening identified 8 compounds with a hit rate of 0.47% and a robust Z′ discrimination factor (0.68 ± 0.1). Of these, Brefeldin A (BFA) was the most successful at inducing lipid accumulation and was used to evaluate secondary screens including measuring levels of fatty acids, photosynthetic pigments, proteins and carbohydrates. The effectiveness of BFA was confirmed in *Chlorella sorokiniana* UTEX 1230. This study demonstrates the power of chemical genomics approaches in biofuel research.
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1. Introduction

In recent years, the microalgae, a diverse group of photosynthetic organisms, have been proposed as a feedstock for biofuel production over traditional crops [1]. Algal niche habitats include marine environments, freshwater, desert sands and more extreme environments such as hot springs and cold environments [2]. Algae also demonstrate a physiological plasticity allowing them to adapt to different environmental conditions. This may be attributed, in part, to their ability to synthesize a complex complement of lipid species, as well as several unusual protective compounds [3]. Eukaryotic microalgae accumulate storage lipids (i.e. triglycerides (TAG)) when subjected to different forms of stress such as nutrient starvation (e.g., nitrogen or phosphorous). Nitrogen starvation has been well documented to induce TAG accumulation widely, however, these conditions eventually terminate growth [4–7]. Similar TAG accumulation was observed following phosphorous limitation in *Pavlova lutheri*, *Isochrysis galbana* and *Phaeodacylum tricornutum*. In contrast, phosphorous limitation was correlated with decreased lipid contents in *Nannochloris atomus* [8]. Temperature stress generally effects the fatty acid (FA) composition of microalgae without increasing lipid accumulation [9]. At high temperatures saturated fatty acids (SFAs) are increased relative to unsaturated fatty acids (UFAs) while, at low temperature UFA is increased relative to SFA. Salinity has also been reported to induce lipid accumulation in microalgae as observed in *Dunaliella teritolecta*. In this case TAG content increases when salt concentration is increased up to 4 M coincident with increasing total SFA and monounsaturated FAs (MUFAs) and a reduction in polyunsaturated FAs (PUFAs) [10]. There are also reports that alkaline pH stress leads to TAG accumulation in *Chlorella* CHLOR1 independent of nitrogen or carbon limitation [11]. Additionally, heavy metals including cadmium, iron, zinc and copper induce lipid accumulation in *Euglena gracilis* [12]. Each of these stress conditions commonly terminates growth and reduces photosynthetic complex components coincident with TAG synthesis and storage. In the present work, we sought to develop a screen useful to identify small compounds in a high throughput format that could induce TAG production in algae while maintaining growth states more comparable to non-stressed conditions.

High-throughput screening (HTS) of chemical compounds to identify modifiers of molecular targets and cellular processes has become a central component of the drug discovery process. This approach, referred to as chemical genomics, is a synthetic ligand-driven method that is directed to alter specific cellular metabolic activities [13–15]. This approach has been previously used in a comprehensive portfolio of applications such as the identification of inhibitors of cancer stem cells [16], inhibitors of enzymes [17], identification of modulators of fat storage in *Caenorhabditis elegans* [18], and inhibitors of fatty acid uptake into cells [15,19]. These types of approaches have not been widely adopted in eukaryotic green algae, yet there are several key studies attesting

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to the power of this approach. In *Chlamydomonas reinhardtii*, a HTS was used to identify small molecule modulators of growth, motility and photosynthesis [20]. In studies addressing lipid metabolism in four strains of oleaginous microalgae (*Nannochloropsis salina*, *Nannochloropsis oculata*, *Nannochloris* sp., and *Phaeodactylum tricornutum*), a small library of known kinase inhibitors, fatty acid synthetase (FAS) inhibitors and oxidative signaling molecules was used in phenotypic screening to identify classes of compounds that increase growth and lipid production [21]. There had been concerns that the hydroxyproline rich cell wall of *Chlamydomonas* might not amenable for small molecule transport across the cell wall, but recent studies have shown this does not pose a significant barrier [20].

In the present work, we developed a small molecule phenotypic screen to identify compounds that induced lipid accumulation while maintaining growth and minimizing induction of stress response pathways. To evaluate this screening method, we employed two test libraries obtained from the National Cancer Institute (NCI), Diversity Set III and Natural Products Set II. Our results demonstrate that a single assay platform based on a classical live cell drug screening approach can be used to identify small chemical inducers of lipid accumulation. The simultaneous screening for both lipid and biomass accumulation in the presence of compound is ideal and will aid in the elimination of those that induce stress response pathways. Employing a small library design to test these high-throughput screening methods, we were able to demonstrate the feasibility and power of this approach with the eventual goal of screening large chemically diverse libraries to ultimately yield viable candidates that induce lipid accumulation with minimal impact on biomass accumulation.

2. Materials and methods

2.1. Chemicals and materials

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Nanopure water at 18 Ω was obtained from Milli-Q Millipore (Millipore, Milford, MA). Clear transparent 96-well U-bottom plates were used for growing cells and black-walled flat bottom plates for fluorescent assays were obtained from BD Falcon.

2.2. Organism and growth conditions

C. reinhardtii CC125 wild type strain was obtained from the *Chlamydomonas* stock center (http://www.chlamy.org/). Prior to screening, cells were maintained in Tris Acetate Phosphate (TAP) media at 25 °C with a photon flux density of 54 μmol m^{-2} s^{-1} in 250 mL Erlenmeyer flasks with a rubber stopper adapted for facilitating gas exchange. Ammonium chloride was omitted to yield TAP N− when nitrogen limitation was required to induce lipid accumulation. Flasks were maintained in a horizontal orbital shaking growing chamber (120 rpm; Innova 43, New Brunswick). For screening, cells were dispensed at low cell density to 96-well microtiter plates as detailed below. Similarly, *Chlorella sorokiniana* UTEX 1230 was maintained in TAP media and grown as detailed above.

2.3. Screening library selection

Two compound-screening libraries were obtained from the open chemical repository collection of the National Cancer Institute (NCI). The libraries consisted of aliquots of Diversity Set III and the Natural Products Set II in 96 well microtiter plates. The Diversity Set III consists of 1597 compounds whereas the Natural Products Set II consists of 120 compounds. The compounds in each well were reconstituted using 19 μL of DMSO to obtain a final concentration of 10 mM. Diversity Set III includes a set of compounds that can be used to generate structure-based hypothesis testing for screening methods development as employed herein. These compounds include pharmacores that are relatively rigid, have less than 5 rotatable bonds and have pharmacologically desirable properties. Many of the compounds have well known chemical properties and biological activities.

2.4. Optimization of Nile Red staining

2.4.1. NR concentration optimization

Cells were pre-grown to mid-log phase, centrifuged (5 min; 3000 ×*g*), and resuspended to an OD_{550} of 0.5 in TAP media for negative (Neg) controls. Controls for lipid accumulation were grown in N− TAP (Pos) and were then rinsed three times and then finally resuspended to OD_{550} of 0.5, all in TAP N− media. In both conditions, cells were cultured for 72 h, harvested, centrifuged and plated in a 96-well plate to a final optical density (OD_{550}) of 0.5. To assess optimal lipid staining, Nile Red (NR) (9-(diethyl amino)benzo[a]phenoxazin-5(5H)-one (Sigma-Aldrich)) was prepared as a stock solution at a concentration of 1.0 mg/mL in dimethyl sulfoxide (DMSO) and added to give the desired final concentration as detailed in Section 3. Plates were incubated either at room temperature (22 °C) or at 37 °C in dark for 30 min with or without shaking. NR fluorescence was measured in arbitrary units at 485/20 (Ex) and 590/35 (Em) using a BioTek Synergy Plate Reader.

2.4.2. DMSO concentration optimization

To determine the optimal DMSO concentration allowable for NR staining of lipid droplets in algal cells, 200 μL of cell suspension with an approximate cell density of 0.5 OD_{500} was used. Dilutions of NR were made to give stock concentrations ranging from 50 μM to 3 mM. The appropriate volume of NR stock was adjusted with DMSO to give the desired final concentrations of NR and DMSO.

2.5. Final screening procedure defined

Cells were grown to mid-log phase (0.5 OD_{550}), harvested by centrifugation, washed twice with TAP media, and then diluted further to give a final optical density OD_{550} of 0.2. Cells were then seeded in a 96-well plate at 200 μL per well and 2 μL of compound solution (stock concentration 1 mM in DMSO) was added to give 10 μM f.c. Separate aliquots of cells and compounds were dispensed using an Eppendorf epMotion 5075 liquid handling robot. The wells in the first column of each plate contained cells alone in TAP media (TAP N+) without compound and served as a positive control for growth and negative control for lipid accumulation (Neg), while the last column of the plate contained cells in nitrogen free TAP media (TAP N−) at a density of 0.5 OD_{550} to serve as the positive control for lipid accumulation (Pos). The latter did not increase significantly in cell density over the course of the experiment due to the lack of nitrogen, thus initial seeding was at a higher cell density. The plates were covered with gas permeable adhesive film (BreathEasy, Diversified Biotech) and incubated in 50–55% humidified atmosphere at 25 °C with a photosynthetic photon flux density of approximately 50–55 μmol m^{-2} s^{-1}. Incubations were continued for 72 h prior to assessment of growth at OD_{550} and assay for lipid accumulation using 10 μM NR and 10% DMSO.

2.6. Data analysis

After removal of the subset of compounds that restricted growth, the raw intensity data for the NR fluorescent signal of each well was background corrected by subtraction of the median intensities across all control wells on the same plate. The background corrected data was used for determining the Z-factor of the plate [22]. The fold-change was calculated by taking the ratio of the NR fluorescence intensity obtained for the treated sample divided by the mean value obtained for the negative control samples (TAP N+). Compounds were identified as hits when the Z-factor [22] of the plate was greater than 0.5 and

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