ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

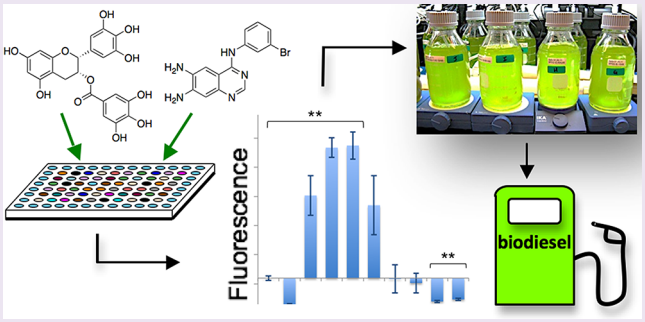
Phenotypic Screening with Oleaginous Microalgae Reveals Modulators of Lipid Productivity

Annaliese K. Franz,* Megan A. Danielewicz, Diana M. Wong, Lisa A. Anderson, and Jordan R. Boothe

Department of Chemistry, University of California, Davis, California 95616, United States

Supporting Information

ABSTRACT: Here we describe the first phenotypic screening with microalgae to study lipid metabolism and to discover organic small molecules as chemical triggers that increase growth and lipid production. A microplate assay has been developed for analysis of intracellular lipids using Nile Red fluorescence in order to screen a collection of diverse bioactive organic molecules (e.g., kinase inhibitors) with four strains of oleaginous microalgae (*Nannochloropsis salina*, *Nannochloropsis oculata*, *Nannochloris* sp., and *Phaeodactylum tricornutum*). Several small molecules identified in microplate screening increased lipid productivity >200% without decreasing growth and biomass production. Selected compounds were further investigated in the context of larger batch culture experiments (e.g., 500 mL) and demonstrated to increase lipid levels (up to 84%) while maintaining or increasing the specific growth rate. Bioactive molecules such as forskolin and quinacrine were identified as promising probes of microalgae lipid pathways. We have also determined that common antioxidants such as epigallocatechin gallate and butylated hydroxyanisole (BHA) increase lipid productivity and may represent new probes of oxidative signaling pathways for photooxidative protection.



As the world's largest group of photosynthetic organism, microalgae have been identified as a viable feedstock for biofuels due to their efficient abilities to convert sunlight and CO_2 to biomass, thrive in saline water, and grow on non-arable land.[1−3] Several oleaginous strains of microalgae have been identified to produce naturally high levels of lipids (e.g., 20−75% dry mass) in the form of triacylglycerols (TAGs), which can be converted for use as biodiesel via a transesterification process.[3] Microalgae are considered to be superior oil producers compared to terrestrial competitors (e.g., palm, rapeseed, jatropha, soybean) because fewer resources are devoted to the synthesis of structural components such as cellulose and lignin.[4] However, for microalgae to become more economically competitive as a feedstock, it is necessary to improve the understanding of microalgae metabolic pathways and increase oil content.

Several methods have been reported for increasing lipid production in microalgae, including nutrient limitation,[5] heterotrophic growth conditions,[6] and genetic engineering methods.[7] While nitrogen-deficient conditions are known to provide an increase in the lipids produced per cell in multiple microalgae strains, there is an overall decrease in the growth and cell mass produced.[8] Although limited examples of genetic transformation have been reported for microalgae, successful examples have been reported using genetic manipulation to increase production (and/or alter the fatty acid composition) of plant oil in organisms such as *Brassica napus* and *Arabidopsis thaliana*.[9] Genetic manipulation of ACCase activity in rapeseed plants resulted in a 5% increase in seed oil content.[10] Overexpression of KAS III has been shown to increase the proportion of palmitic acid (16:0) but led to an overall decrease in TAG content by 5−10%.[11] Overexpression of cytosolic glycerol-3-phosphate dehydrogenase (gpd1) in *Brassica napus* led to a 40% increase in seed oil content.[12,13] Genetic alteration of the acyltransferases in *A. thaliana* led to a 10−21% increase in oil production.[14,15] Initial studies that increased ACCase activity in transformed microalgae did not result in any increase in lipid production.[3] To date, there have been very few positive results reported using genetic engineering to alter lipid metabolism in microalgae.[7,9] One promising current direction is the deletion of starch biosynthesis genes in combination with nitrogen starvation/limitation, which has been shown to increase lipid production in non-oleaginous *Chlamydomonas reinhardtii* as a model organism.[16−18] RNAi methods have also been useful to manipulate the gene expression in *C. reinhardtii*, enabling a reverse genetic approach to probing gene function,[19,20] but have not been applied to lipid metabolism pathways.

An alternate approach to modulate lipid pathways is to conduct screens of bioactive small molecules with commercially viable oleaginous microalgae to identify chemical triggers that directly elicit the desired phenotype by modifying protein function in real time, rather than genetic mutation methods to

Received: October 24, 2012
Accepted: March 12, 2013
Published: March 22, 2013

disrupt gene function.[21−23] This phenotypic approach does not require knowledge of the small molecule's binding target and can also effect combinations of pathways involved in lipid metabolism and catabolism that may not be identified using other methods. This chemical strategy has not been utilized previously with microalgae for lipid production; however, the use of small molecules in the nanomolar or micromolar concentration range is well established for screening in the pharmaceutical industry with mammalian models of disease biology. Previous phenotypic screening has also been developed with *E. coli*, yeast, and other microorganisms.[22,24−26] Additionally, the effects of small molecules have been investigated on the development of plants such as *Arabidopsis*.[27] Recently, *C. reinhardtii* has also been used as a model for screening anticancer drugs[28] and inhibitors that regulate flagella.[29] The use of bioactive molecules can also provide the opportunity to optimize microalgae strains using chemical (random) mutagenesis.

Herein, we report new chemical triggers that increase lipid productivity in microalgae while either increasing or maintaining growth and cell density. Compounds have been specifically selected to enhance potential for industrial applications by selecting molecules with picomolar to micromolar activity for characterized target classes (e.g., in yeast and mammalian cells), such as various kinase inhibitors, fatty acid synthase (FAS) inhibitors, elicitors used in plant biology, and oxidative signaling molecules (Supplementary Table S1). Our results show that these bioactive chemical triggers identified in microplate screening can be used in larger cultures to increase both the biomass (based on cell density and dry weight) and lipid levels (based on extract weight) in microalgae. The lipid levels we observe match the lipid content per cell that is typically observed for nitrogen-deficient conditions, but without an overall decrease in cell mass. Using bioactive small molecules in 500 mL cultures, we have identified conditions that demonstrate the first proof of concept for this approach and increase lipid levels up to 84%. Furthermore, we have demonstrated that several compounds are relevant and economically viable for industrial applications based on a cost analysis of quantities for large-scale microalgae production.

RESULTS AND DISCUSSION

Screening for Modulators of Lipid Production in Microalgae. We have designed a three-phase screening process to identify small molecule probes and chemical triggers to increase growth and lipid levels in oleaginous microalgae relevant for biofuel production (Figure 1). The initial screening phase utilizes 96-well microplates to screen a pilot collection of diverse bioactive molecules selected on the basis of characterized target classes (e.g., in plants, yeast or mammalian cells; Supplementary Table S1) with multiple industrially relevant microalgae strains (selected based on high-lipid content). Here we have initiated screening with a pilot collection of bioactive molecules in order to screen two or more concentrations with four oleaginous microalgae strains. Microalgae species evaluated in this assay include *Phaeodactylum tricornutum*, *Nannochloropsis salina*, *Nannochloropsis oculata*, and *Nannochloris* sp., which have been previously described as valuable for commercial biofuel applications. The microplate assay monitors growth using absorbance and chlorophyll fluorescence measurements during all phases, followed by the addition of a lipophilic dye (Nile Red, 9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) to measure intracellular lipid

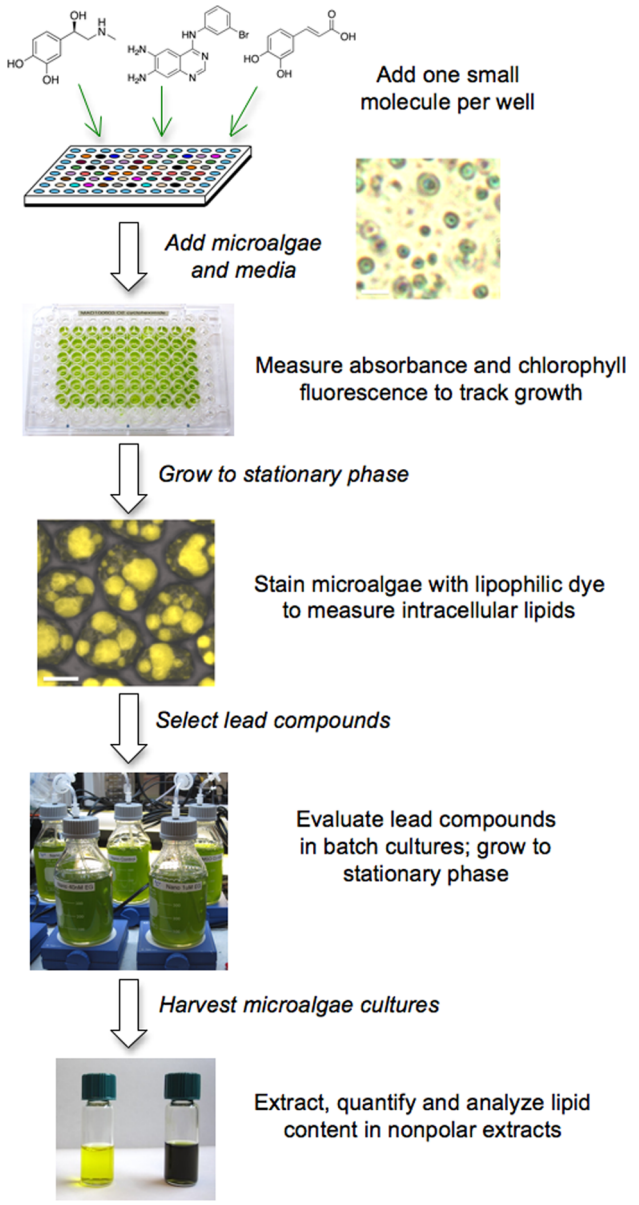


Figure 1. Overview of screening process to identify small molecules that increase lipid production in microalgae.

levels in stationary phase.[30] The second phase involves further dose−response screening in order to confirm the activity of lead compounds that have been identified in microplates. The final screening phase investigates the activity of lead compounds in larger 500-mL cultures to quantify and compare lipid levels and composition using traditional methods of lipid analysis, such as gravimetric analysis, ^{1}H NMR spectroscopy, microscopy, and mass spectrometric analysis (MALDI-TOF of TAGs and GC−MS of FAMEs).[31] These methods also facilitate the "pre-characterization" of fuel properties in order to attain desired properties of biodiesel (such as volatility and viscosity) where small changes resulting from unsaturation or chain length can have a substantial effect on fuel properties (e.g., cloud point, oxidative stability).[26,32] Several lead compounds were also selected for screening with water as a delivery vehicle, instead of DMSO, due to the relevance for industrial-scale applications.

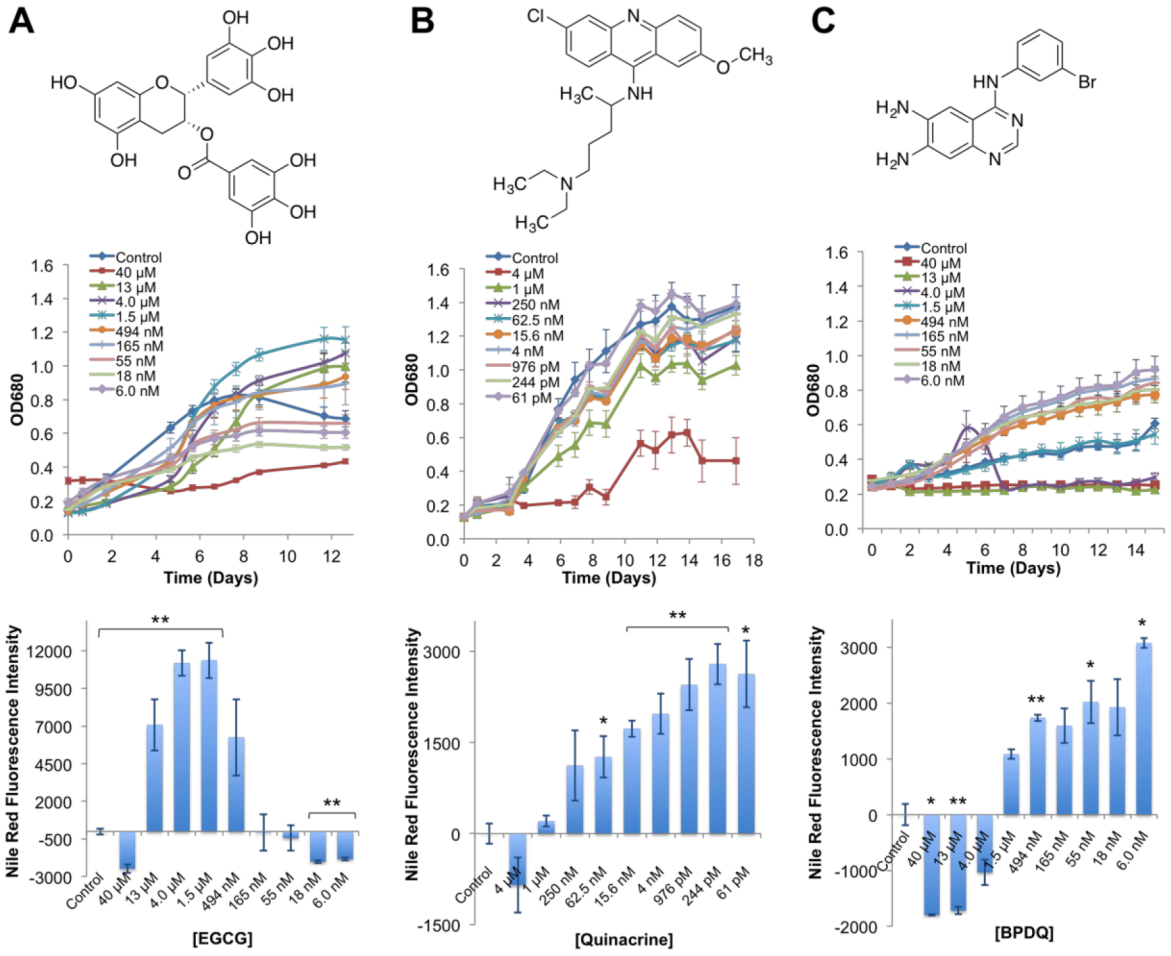


Figure 2. Dose–response effects for microalgae biomass concentration and intracellular lipid levels in microplates. Intracellular lipid levels measured by Nile Red fluorescence on day 3 of stationary phase, which differs for each microalgae strain. (A) Dose–response effects of (−)-epigallocatechin gallate (EGCG) with *N. salina*. (B) Dose–response effects of quinacrine with *N. oculata*. (C) Dose–response effects of BPDQ with *N. salina*. Data points and error bars (SEM) in optical density and Nile Red fluorescence measurements represent a mean of replicates. [A: $n = 6$, B: $n = 4$, C: $n = 3$ or $n = 2$ (4.0 and 1.5 μM]. * = $P < 0.05$, ** = $P < 0.01$, two-tailed t test.

Initial small molecule screening investigated 432 unique culture conditions using a collection of 54 bioactive molecules screened in all four microalgae strains at 20 μM and 200 nM concentrations. This preliminary screening evaluated commercially available bioactive small molecules with characterized target classes, such as various kinase inhibitors, fatty acid synthase (FAS) inhibitors, plant hormones, and oxidative signaling molecules (Supplementary Table S1). Many of the compounds selected were nontoxic to enhance long-term commercial potential for use in large-scale pond production of microalgae. Optimization of the microplate assay required the addition of sodium bicarbonate or soil extract as supplemental carbon sources (Supporting Information) because limited air exchange occurs in microplates, and air or CO_2 bubbling was not feasible.[33,34] The addition of sodium bicarbonate as an external inorganic carbon source ensured consistent growth conditions in microplates to allow for comparative studies based on compound treatment.

We have observed five outcomes based on growth and lipid phenotypes, depending on both the compound structure and concentration: (1) increase in both growth and lipid production; (2) increase in growth but decrease in lipid production; (3) decrease in growth but increase in lipid production; (4) decrease in both growth and lipid production; (5) no effect on growth or lipid production. The first three outcomes are the focus of this study. Statistical analysis was performed on approximately 800 microplate cultures per species. Average and standard error of the mean were used to compare lipid production based on Nile Red fluorescence intensities. Compounds with Nile Red fluorescence intensity consisting of $p < 0.05$ in an independent two-tailed t test were considered statistical significant (Supplementary Table S2). Based on this error analysis, microplate assay results with a lipid increase >20% (regardless of growth rate) were considered to be positive results (i.e., hits) for the first phase of the assay. A complete summary of molecules with >20% increase of intracellular lipids based on Nile Red fluorescence intensity is found as Supplementary Table S3. Control experiments were performed to ensure that there were no false positives based on compound autofluoresence and also that compounds do not interfere with the quantification of lipid levels (i.e., enhance or quench the fluorescence) using the Nile Red dye (see Supporting Information).

Several classes of molecules were identified as having a consistent effect to increase intracellular lipid levels. Several strains of green microalgae responded positively to certain compounds (e.g., cAMP, forskolin, and quinacrine), suggesting that there are conserved metabolic targets among different

dx.doi.org/10.1021/cb300573r | *ACS Chem. Biol.* 2013, 8, 1053–1062

Table 1. Summary of Compounds Affording >50% Increase of Intracellular Lipid Levels in Microplate Dose−Response Screening Relative to DMSO Control ($p < 0.05$)[a]

microalgae	compound[b]	concentration	% lipid increase based on Nile Red fluorescence intensity[c]
N. oculata	baicalein	25.2 pM	52
	baicalein (water)	40 μM	76
	curcumin	75.7 pM	49
	quinacrine	244 nM	106
	SB202190[d]	400 fM	95
N. salina	arctigenin	370.4 nM	64
	atrazine	4.6 nM	53
	BPDQ	6.0 nM	130
	CDK4 inhibitor I	40 μM	49
	epigallocatechin gallate	1.48 μM	217
	esculetin	6.13 nM	62
	forskolin	4.6 nM	66
	gossypol	6.13 nM	103
	indomethacin	123.5 nM	61
	JZL 184 hydrate	40 μM	119
	methyl jasmonate	13 nM	85
	PTP inhibitor II	22.7 pM	54
	zeatin	4.6 nM	64
Nannochloris sp.	bisindolylmaleimide (water)	49 nM	141
	FAAH inhibitor I (water)	0.6 nM	189
	FAAH inhibitor II (water)	0.6 nM	182
	indomethacin (water)	5.5 nM	95
	ketoconazole (water)	148 nM	94
	piceatannol	6.10 nM	71
	quinacrine (water)	49 nM	136
P. tricornutum	arctigenin	41.2 nM	61
	BPDQ (water)	400 fM	63
	caffeic acid (water)	4 μM	94
	cAMP	370.4 nM	66
	CDK2 inhibitor 2	40 μM	347
	CDK4/6 I4	10 μM	50
	cycloheximide[d]	400 nM	408
	naphthyl acid phosphate (water)	681 pM	51
	resveratrol	10 nM	61
	zeatin	4.6 nM	114

[a]Increases in Nile Red fluorescence intensity were based on three or more replicates with $p \leq 0.05$. Depending on the experiment, replicates were tested in different 96-well microplates or within the same microplate. The p-values were calculated utilizing a two-tailed independent t test. [b]Solutions of DMSO are used for compound delivery, except for cases where water is indicated in parentheses. Compounds were added at the start of analysis on t = day 0. [c]Refer to Supplementary Table S4 for more information regarding additional compound concentrations, p-values and number of replicates. [d]Compound was added at exponential phase, t = day 7.

strains. However, compounds such as (−)-epigallocatechin gallate (EGCG), PTP inhibitor II, and cycloheximide afforded varied responses based on the microalgae strain, which could indicate that regulation of some biological pathways occurs by different mechanisms in different strains. On the basis of the results of our initial microplate screening, the following conclusions can be made: (1) adding supplemental carbon sources (e.g., sodium bicarbonate) was important for microalgae growth in microplates to successfully develop the microplate assay; (2) the Nile Red fluorescent assay is a rapid and effective way to perform chemical genetic screening and compare intracellular lipid content in a microalgae microplate assay; (3) generally, *N. salina* and *Nannochloris* sp. exhibited more positive increases in intracellular lipid levels, whereas *N. oculata* and *P. tricornutum* exhibited fewer positive results; (4) chemical triggers identified to increase intracellular lipid levels for *P. tricornutum* were generally distinct from molecules identified for green microalgae strains. Since we have also identified many molecules with negligible effects on the growth and lipid levels of microalgae, this suggests that microalgae are robust and have adapted to being exposed to "contaminants" in their environment.

Dose−Response Screening of Lipid Modulators for Microalgae in Microplates. Based on compounds promoting >20% intracellular lipid increase based on Nile Red fluorescence intensity relative to the control, a series of different compound/strain combinations were identified to investigate for concentration-dependent activity. Additional related compounds were also selected on the basis of the target classes or known functions of these lead compounds, such as modulators of lipoxygenase activity, lipase activity, plant hormone activity, protein kinase activity, and oxidative signaling activity.[35−37] In total, 42 compounds were evaluated in dose−response screening in four microalgae strains at nine concentrations (ranging from 40 μM to 2 pM) to further

Table 2. Chemical Triggers Increasing Lipid Productivity in Microalgae in 500 mL Cultures[a]

microalgae	compound	specific growth rate ($days^{-1}$)[b]	lipid content (% w/w)[c]	lipid productivity ($mg\ L^{-1}\ day^{-1}$)	change in lipids (%)[d]
Nannochloris sp.	control	0.17 ± 0.03	–	3.0 ± 0.9	
	DMSO (0.4%)	0.21 ± 0.11	–	2.6 ± 0.2	−16
	10 μM forskolin	0.15 ± 0.05	–	4.5 ± 1.7	51
	400 nM cAMP	0.17 ± 0.01	–	4.8 ± 0.5	62
	40 nM quinacrine	0.18 ± 0.01	–	4.2 ± 1.1	31
	40 nM orlistat	0.20 ± 0.01	–	5.1 ± 1.5	72
	40 nM EGCG	0.17 ± 0.03	–	4.3 ± 0.8	39
	4 nM SB202190	0.17 ± 0.02	–	3.0 ± 1.7	3
	4 nM SB202190 (exponential addition)	0.15 ± 0.08	–	4.0 ± 1.6	38
N. oculata	control	0.16 ± 0.04	16.1 ± 6.6	3.5 ± 0.7	
	DMSO (0.4%)	0.20 ± 0.07	18.6 ± 7.8	4.8 ± 0.6	39
	4 nM forskolin	0.17 ± 0.02	25.6 ± 1.8	6.4 ± 1.2	84
	400 nM cAMP	0.19 ± 0.03	22.3 ± 11.0	4.4 ± 1.8	51
	400 nM quinacrine	0.18 ± 0.04	16.7 ± 4.9	5.2 ± 0.6	51
	4 μM EGCG	0.17 ± 0.03	20.6 ± 6.9	4.9 ± 1.7	41
N. salina	control	0.23 ± 0.06	23.6 ± 9.0	4.7 ± 1.4	
	DMSO (0.4%)	0.26 ± 0.07	24.3 ± 7.5	6.5 ± 1.7	25
	4 μM cAMP	0.22 ± 0.03	28.6 ± 10.7	7.9 ± 3.4	46
	40 nM quinacrine	0.25 ± 0.02	26.5 ± 3.9	4.5 ± 1.0	6
	40 μM EGCG	0.22 ± 0.06	23.1 ± 10.6	6.3 ± 2.2	18
	4 μM EGCG (in water)[e]	0.24 ± 0.01	32.4 ± 0.1	7.1 ± 1.2	46
	40 nM propyl gallate	0.23 ± 0.002	25.9 ± 11.4	8.3 ± 1.7	67
	4 nM BHA	0.23 ± 0.002	28.8 ± 11.4	8.1 ± 0.6	63
P. tricornutum	control	0.27 ± 0.09	15.6 ± 6.1	3.9 ± 1.7	
	DMSO (0.4%)	0.27 ± 0.08	21.5 ± 5.9	4.1 ± 1.6	−1
	76 pM gossypol	0.21 ± 0.05	24.4 ± 2.7	5.6 ± 1.9	27
	40 nM cAMP	0.26 ± 0.19	16.7 ± 0.7	5.3 ± 1.0	28
	120 nM AICAR	0.34 ± 0.04	22.7 ± 1.3	7.0 ± 0.9	45
	4 μM EGCG (in water)[e]	0.19 ± 0.02	17.2 ± 1.3	4.4 ± 2.4	6

[a]A comparison of dry weight and lipid extracts for 500-mL microalgae cultures treated with bioactive small molecules vs control cultures with and without DMSO. All cultures were performed with three or more replicates unless otherwise indicated. [b]Specific growth rate was calculated during mid-exponential growth phase. Refer to Supporting Information for details. [c]Lipid content determined with dried lipid extract and dry biomass (see Supporting Information and Supplementary Table S5). All data indicate the average with the standard deviation denoted. Standard deviation was calculated from within individual batch culture sets. See Supporting Information for full experimental details. Due to small cell size, *Nannochloris* sp. samples were not fully dried, and therefore, the w/w analysis of lipid content was not directly calculated. [d]The change in lipid is calculated on the basis of gravimetric analysis relative to the averaged control without DMSO. [e]Two replicates performed.

characterize the activity of these chemical triggers to effect growth and intracellular lipid levels.

From this dose−response microplate screening based on Nile Red fluorescence intensity, we identified 12 compound/strain combinations that increase intracellular lipid levels >100%, with three compounds affording increases in intracellular lipids of 200−400%. Many compounds showed activity in microalgae at nanomolar concentrations and several showed activity in multiple microalgae strains. For example, quinacrine, an NF-κB inhibitor and p53 activator, demonstrates activity to increase intracellular lipids >100% compared to the control (based on Nile Red fluorescence intensity) in *N. oculata* and *Nannochloris* sp. (in water). (−)-Epigallocatechin gallate (EGCG), a catechin found in green tea, also showed intracellular lipid increases >100% at several concentrations. Figure 2 shows examples of dose−response screening results for cell growth and intracellular lipid measurements for (−)-EGCG, quinacrine, and BPDQ in *N. salina*, *N. oculata*, and *N. salina*, respectively (see also Supplementary Figure S7 for additional examples). In cases where compounds exhibited nanomolar activity for effects on growth or intracellular lipid levels, additional concentration ranges were evaluated to determine the most promising concentrations for testing in larger batch culture experiments. A summary of microalgae species and concentrations of lead compounds exhibiting the greatest effect on lipid levels is provided in Table 1 (see also Supplementary Table S4). The basic trends observed for dose−response screening followed the examples shown in Figure 2 (and Supplementary Figure S7).

We also demonstrated the importance of temporal effects for small molecule screening by adding compounds at different microalgae growth phases (e.g., initial vs exponential phase), an option that is not typically available when gene function is disrupted using classical genetic manipulation. Initial compound screening was performed with compounds added to the microplate before the start of the assay (i.e., at $t = 0$). A second round of microplate screening was performed where microalgae cultures were treated with lead compounds during the exponential phase of growth (t = day 7) to evaluate potential temporal effects. Small molecules that demonstrated growth-inhibitory properties when added at $t = 0$ were of particular interest to evaluate temporal effects because it was envisioned that these compounds may mimic nutrient-limiting stress conditions and shift the metabolic pathways to increase lipid levels without having detrimental growth effects as a result of being introduced later in the growth cycle. The temporal effects

dx.doi.org/10.1021/cb300573r | ACS Chem. Biol. 2013, 8, 1053−1062

for cycloheximide, a protein synthesis inhibitor, and SB202190, a MAP38 kinase inhibitor, were particularly noteworthy (Supplementary Figure S5). When microalgae cells were treated with cycloheximide in the initial phase at concentrations >1 μM, an inhibitory effect on cell growth was observed (based on low absorbance compared to control in all four species) and only a minimal increase in lipid levels was observed. However, when cycloheximide was added during the exponential growth phase, an increase in lipid levels (up to 400%) was observed over a greater concentration range for *P. tricornutum*. The addition of SB202190 in exponential growth phase also afforded a more significant increase in the intracellular lipid levels for *N. oculata* (where an increase from 45% to 95% is observed based on Nile Red fluorescence intensity at >0.040 nM concentrations). While these results highlight the potential impact of performing compound treatment at various growth phases, comparing temporal effects may also help elucidate the mechanism of action for a compound.

Translating the Effects of Chemical Triggers to Increase Lipid Levels in Larger Cultures. On the basis of dose−response screening results, candidate compound/strain combinations were selected for screening in 500-mL batch cultures to confirm their effectiveness in the context of larger scale microalgae batch cultures where lipid extracts can be quantified using gravimetric analysis. Selection of compound/strain combinations was based on demonstrated increases in lipid levels >50% based on Nile Red fluorescence intensity (p-value <0.05) in dose−response screening or if positive results were observed in more than one microalgae species. With this size culture, secondary assays were also performed to analyze lipid composition using ^{1}H NMR spectroscopy, MALDI-TOF, and GC−MS (see Supplementary Figures S6−8 for examples). Batch culture screening was performed with four microalgae strains, but the most significant effects of compounds on lipid production were observed in green microalgae strains, highlighted in Table 2. Compared to microplates, microalgae grown in batch cultures are aerated, mixed, and exposed to light and other contaminating organisms more similarly to bioreactors or outside growth ponds used for industrial microalgae production, which makes them more representative to predict success in industrial applications.

Positive increases in growth and lipid levels (based on cell density, biomass, and gravimetric analysis on neutral lipids extracts) were identified with bioactive molecules such as forskolin, cAMP, quinacrine, orlistat, and EGCG. Most compounds were effective when added at the beginning of the culture growth, but SB202190 afforded a 38% increase in lipid levels in *N. oculata* batch culture only when added during the exponential growth phase as expected based on our previous temporal screening experiments in microplates (Supporting Information Figure S8). Forskolin and cAMP were selected due to their known activity as protein kinase activators, with forskolin known to increase cAMP levels and cause lipolysis.[38] After confirming positive preliminary results for forskolin in the initial microplate screening, both forskolin and cAMP were investigated in parallel for batch culture experiments. In batch cultures, forskolin led to an 84% increases in lipid levels in *N. oculata,* and a smaller increase was also observed in *Nannochloris* sp. cAMP also increased lipid levels by 28−62%, depending on the microalgae strain. Quinacrine, a known inhibitor of PLA_2 and NF-κB, and an activator of p53, showed moderate lipid increase activity (31−51%) in two microalgae strains. It is notable that these cultures maintain or increase growth (i.e., cell density) while maintaining lipid production (Figure 3). The fact that growth

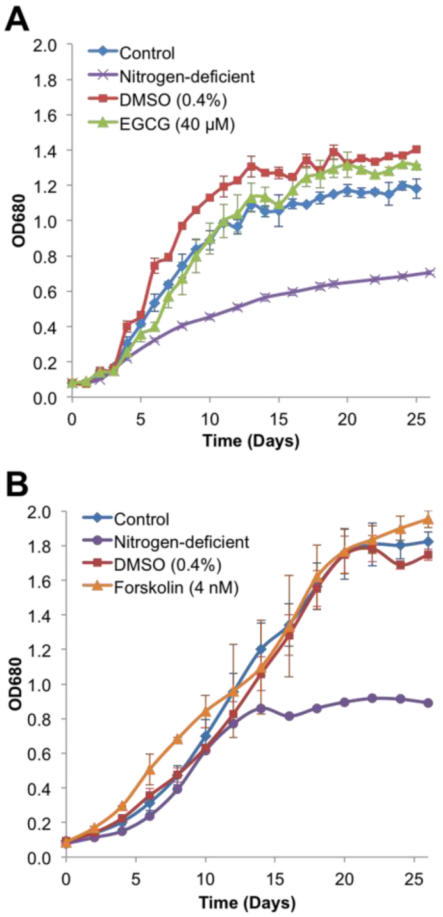


Figure 3. Comparison of microalgae growth for trigger-induced and nitrogen-deficient conditions in 500-mL batch cultures for (A) *N. salina* and (B) *N. oculata*. Data points represent the mean of replicates with error bars (SEM) [A: control, n = 6; DMSO (0.4%), n = 5; EGCG 40 μM, n = 4; nitrogen deficient, n = 2. B: control, n = 4; DMSO (0.4%), n = 3; nitrogen deficient, n = 2; forskolin (4 nM), n = 2].

is maintained in the presence of these chemical triggers is in contrast to the effects observed and reported for the nitrogen-starvation conditions, which are often utilized as a model system to study increased lipid productivity.[3] Nitrogen-deficient conditions are known to reduce cell division, while increasing overall lipid productivity per cell compared to normal growth conditions.[5,8] Chemical triggers such as EGCG and forskolin meet the goal of increasing overall lipids without a decrease in cell mass (Figure 3). Analysis of the lipid composition for compound-treated cultures showed no significant change in fatty acid profiles based on ^{1}H NMR spectroscopy, MALDI-TOF, or GC−MS analysis (See Supporting Information). Synergistic screening of compounds using nitrogen-limited growth conditions may also provide additional opportunities to increase lipid levels with this small molecule activation strategy. The synergistic effects of sodium bicarbonate in addition to chemical triggers, may partially account for the difference between results observed for microplate and batch culture lipid levels, and also indicate an important area for additional exploration.

dx.doi.org/10.1021/cb300573r | *ACS Chem. Biol.* 2013, 8, 1053−1062

Table 3. Cost Analysis to Dose a 50,000 L Pond of Microalgae with Optimal Compound Concentrations

compound	price/g[a]	optimal concn identified in screening[d]	desired concn (M)	MW (g/mol)	amount needed	price for 50,000 L pool
EGCG	$1,200.00[b]	4 μM	4.00×10^{-06}	458.40	91.68 g	$110,016.00
cAMP	$107.00	4 μM	4.00×10^{-06}	329.20	65.84 g	$7,044.88
forskolin	$4,950.00[c]	4 nM	4.00×10^{-09}	410.50	0.0821 g	$406.40
BHA	$0.06	4 nM	4.00×10^{-09}	180.24	0.036 g	$0.002
Propyl gallate	$0.09	40 nM	4.00×10^{-08}	212.20	0.4244 g	$0.04
DMSO	$64.16/L	0.04%	–	–	20 L	$1,283.20

[a]Unless otherwise indicated, the price per gram for each compound is based on the direct comparison of pricing from Fisher Scientific or Sigma Aldrich without academic discounts, whichever company provided a more cost-effective pricing for larger scale quantities (e.g., in some cases up to 10 kg). [b]Based on pricing from Axxora for 50 mg. [c]Based on pricing from AK Scientific for 100 mg. [d]Optimal concentration values are based on results with *N. salina*, with the concentrations of forskolin based on results with *N. oculata*.

The use of DMSO as a compound delivery vehicle was generally determined to be important for compounds effects, most notably in the green microalgae strains, which are known to have thick cell walls.[30] DMSO was utilized due to its ability to promote the permeation of solutes as well as the solubility of the majority of bioactive compounds in it.[39,40] DMSO is also known to be a versatile molecule with cryoprotective,[41] antioxidant,[42] and lipid-enhancing properties.[43] DMSO is known to be naturally occurring in microalgae[44] and originates from the enzymatic cleavage product of dimethylsulfoniopropionate (DMSP), dimethyl sulfide (DMS). In microalgae species such as *Thalassiosira pseudonana*, DMSP serves as an antioxidant and cryoprotectant.[45]

While all preliminary compound screening was performed with DMSO stock solutions (with a final volume of 0.4% per culture), lead compounds were also screened with water as a delivery vehicle to determine if the DMSO was contributing to the observed effects. In the green microalgae strains, using water as a delivery agent for compounds often led to minimal effects (<40% increase) on the lipid levels, indicating that the DMSO is essential to facilitate the entry of the compound into the cell or to enhance the effect of the chemical trigger. This was not the case with EGCG, which showed similar effects in *N. salina* when screening either with water or with DMSO, thus indicating that the need for DMSO is dependent on either the compound or the microalgae strain. In the specific case with the diatom *P. tricornutum*, there were several cases where the use of water as a delivery agent afforded an increase in the potency of several compounds, which was attributed to an enhanced sensitivity of the diatom to DMSO. DMSO was independently screened in microplates at concentrations from 0 to 1.4% (v/v) to determine how the vehicle affected microalgae growth and intracellular lipid levels (Supplementary Figure S2). At less than 0.4% v/v, DMSO did not show a toxic or detrimental effect on the growth for any of the four microalgae strains investigated.[39,46] With the addition of higher levels of DMSO (e.g., >0.8% v/v) a more substantial and detrimental effect on growth and cell density was observed for *N. oculata* and *N. salina*. Upon comparison of batch culture control experiments for DMSO (at 0.4% v/v), we observed a 39% and 19% increase in lipid levels with *N. oculata* and *N. salina*, respectively (Table 2). In *P. tricornutum* or *Nannochloris* sp., the addition of 0.4% v/v DMSO generally showed no effect or a small decrease in growth and lipid production.

In all green microalgae strains, we observed that nanomolar or micromolar concentrations of EGCG increased growth and lipid extract levels (Table 2). In the case of *N. salina*, this result was consistent when the compound was delivered using either DMSO or water as the vehicle. Various biological effects have been reported for EGCG, including inhibition of fatty acid synthase, α-glucosidase, and MAP kinase mediated signaling pathways.[47,48] Furthermore, we postulated that the antioxidant action of EGCG could protect cells from lipid peroxidation,[49] which may be involved in modulating photooxidative stress pathways and would therefore reduce photooxidative stress and enhance photosynthetic efficiency.[50,51] Although some information is known in plants, the effects of oxidative stress and the connection to lipid production are poorly understood in microalgae.[52]

We hypothesized that antioxidants such as EGCG may serve as an oxidative signaling molecule to enhance photoprotection and thus may provide a new pathway to investigate for increasing lipid production. To further explore this hypothesis, we investigated the effects of several additional molecules with known antioxidant properties, with a particular focus on readily available derivatives and molecules already used in industrial applications, such as butylated hydroxyanisole (BHA), a preservative used in packaged foods. We first evaluated a series of antioxidants in microplate assays to identify optimal concentration ranges and then moved to larger batch culture experiments. In microplate assays with *N. salina*, both BHA and propyl gallate, a simplified derivative of the EGCG structure, showed positive results with increases in intracellular lipid levels at 40 nM concentration (based on Nile Red fluorescence intensity). The effects of BHA and propyl gallate were further evaluated in batch cultures for *N. salina*, and these effects translated to batch cultures with >60% increase based on gravimetric analysis of neutral lipid extracts.

To assess the industrial viability of using these molecules for growing microalgae in a large-scale pond, we performed a simple analysis to determine the quantity and cost for several lead compounds to dose a 50,000 L pool size (Table 3). While EGCG is not a competitively priced molecule for industrial applications, this initial screening result led us to identify propyl gallate and BHA, which exhibit similar increases in lipid levels and are considerably more affordable for large-scale applications. Performing this simple cost analysis demonstrates that BHA is commercially relevant with a cost of only $0.002 to dose a 50,000 L pond of microalgae. Although the cost and usage of DMSO is undesirable for industrial applications, the overall amount of DMSO could be decreased 10-fold using a more concentrated DMSO stock solution for larger-scale microalgae pools. Furthermore, preliminary results indicate that DMSO is not required for compound delivery and thus the cost and usage of DMSO can be avoided entirely.

In conclusion, we have demonstrated the first example of chemical genetic phenotypic screening with microalgae and have identified several molecules that increase growth and lipid

levels at nanomolar and micromolar concentrations. In order to optimize microalgae lipid production to make algal biodiesel more cost-effective, the ideal conditions will increase the amount of lipid produced per cell while the cell density per culture is simultaneously increased. An increase in lipids, whether by increasing lipid per cell or overall increase in biomass, demonstrates how overall lipid yield can be increased by using a small molecule in nanomolar concentration. Using a novel approach to accomplish this goal, we have demonstrated the first proof-of-concept studies that chemical genetics and phenotypic screening can be used to identify chemical triggers of signaling pathways relevant to increase cell density or lipid levels in several oleaginous microalgae relevant for biofuel feedstock.[3] Our results here confirm that specific small molecules (e.g., forskolin, quinacrine, BPDQ, and EGCG) provide useful modulators to study lipid pathways, as an alternative to the use of nitrogen-limiting conditions, and can lead to the development of chemical triggers to increase biomass and lipid production to advance microalgae biofuel technology.[53] Our initial success with up to 84% increase in lipids in batch cultures represents a significant advance for industrially relevant strains.

Due to the intimacy of the carbon metabolism and photo-oxidative stress along with the redox signal transduction mechanism that regulate carbon metabolism, oxidative signaling molecules, such as antioxidants, kinase activators and inhibitors, can be used to control and improve cell growth while also increasing lipid levels. Testing compound effects across multiple microalgae strains indicates that these molecules may be useful as new probes for lipid synthesis and metabolism and may facilitate the identification of conserved protein targets for genetic engineering. By understanding and controlling lipid-producing pathways, the enhanced production of triacylglycerols from microalgae will make algae-derived biofuels an economically feasible liquid fuel option. Investigations are underway to determine whether these molecules have a general effect on microalgae metabolism or a specific effect on lipid synthesis. Although the direct mode of action for these known bioactive molecules remains to be confirmed in microalgae, these studies highlight the power of using small molecules to modulate microalgae pathways and show significant promising results for commercial applications.[54,55]

METHODS

Microalgae Strains and Maintenance. *Phaeodactylum tricornutum* (UTEX B2089), *Nannochloropsis oculata* (UTEX LB2164), and *Nannochloris* sp. (UTEX LB2055) were purchased from the UTEX Culture Collection of Algae at the University of Texas. *Nannochloropsis salina* (CCMP 537) was purchased from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) [formerly, the Center for the Culture of Marine Phytoplankton at the Bigelow Laboratory for Ocean Sciences (CCMP)]. *P. tricornutum* and *N. salina* were cultured in F/2 medium, and *N. oculata* and *Nannochloris* sp. were cultured in Erdschreiber's medium. Microalgae were maintained at 22 °C on a 16:8 h light/dark cycle and aerated by stirring with bubbling filtered air or by orbital shaking. Refer to Supporting Information for more details.

Microalgae Growth Conditions for Microplate Assay. All wells in the parafilm-sealed clear 96-well microplate contained 100 μL of microalgae cells suspension diluted in 150 μL of appropriate media to obtain a final absorbance of 0.075 (680 nm). *P. tricornutum* and *N. salina* were cultivated by supplementing 1.2 g/L of sodium bicarbonate to the growth media as a carbon source (refer to Supporting Information for more details). Control wells in microplates consisted of microalgae with 0.4% v/v DMSO. Perimeter wells contained no microalgae but were filled with 100 μL water and 150 μL media to prevent edge effects and used for background subtraction.

Addition of Small Molecules for Microplate Assay. Compounds were diluted by serial dilution to desired concentrations starting from a 10 mM compound stock dissolved in DMSO or water. Growth media and 1 μL of compound were dispensed to all wells before the addition of microalgae suspension to minimize the effect of exothermic reaction of DMSO and media. Compounds were assigned randomly within a microplate, but each plate replicate had identical compound position per well. The list of compounds and their manufacturer can be found in the Supporting Information.

Screening for Intracellular Lipids in Microalgae. Microalgae growth was measured on the basis of cell density, which was monitored daily with a multimode UV−vis spectrophotometer and spectrofluorimeter plate reader at absorbance of 680 nm and chlorophyll fluorescence at excitation/emission wavelength of 360/645 nm, respectively. Analysis of intracellular lipids in microalgae was performed using Nile Red fluorescence (530/590 nm) intensity, as described below, after microalgae has grown 3 days in stationary phase, which occurred on day 14 for *N. salina*, *P. tricornutum*, and *N. oculata* and day 21 for *Nannochloris* sp. Nile Red fluorescence intensity values are reported as the difference relative to the DMSO control.

Nile Red Optimized Procedure for Microplate Assay. Intracellular lipid analysis for *P. tricornutum* involved the addition of 25 μL of 1 mg mL^{-1} Nile Red dissolved in acetone followed by a 20-min kinetic fluorescence analysis using a multimode plate reader at excitation and emission wavelength of 530/40 nm and 590/40 nm, respectively. Intracellular lipid analysis in *N. oculata*, *Nannochloris* sp., and *N. salina* require the addition of a 25 μL mixture of 1:1 (v/v) DMSO/media before the addition of Nile Red, and analysis was performed at 35 °C. This microplate method was optimized for our study based on the procedure by Chen et al.[30] The average maximum Nile Red fluorescence intensity from each well was used to compare lipid levels. Background fluorescence intensity of the wells was subtracted. Data analysis was acquired with Gen5 and analyzed with Microsoft Excel. Two methods of lipid analysis were attempted based on comparison with the controls within the same plate and other plate replicates. Refer to the Supporting Information for more details.

Statistical Analysis for Microplate Screening. Reported means, standard deviations, standard errors, and *p*-values were calculated for each compound based on three replicates (one compound per microplate). Error analysis for controls consists of six well replicates per plate all in the same row in addition to an external plate of 54 control wells. In microplates, compounds that caused Nile Red fluorescence intensity readings to significantly deviate from the control ($p < 0.01$) were considered a "hit" (Supplementary Tables S2 and S3). *t* test analysis was based on a two-sided analysis using unequal variance. All statistical analysis and graphs were performed with Microsoft Office Excel.

Screening of Lead Compounds in 500−mL Batch Culture. Batch cultures were set up following similar procedures as in microplates with media, and compound dissolved in DMSO was added before the addition of microalgae suspension. Two milliliters of compound, dissolved in DMSO to desired concentration, was added to cultures at day $t = 0$ (except where indicated for temporal studies) to obtain a DMSO concentration of 0.4% v/v in 500 mL, similar to the conditions in microplates. Cell density was monitored by measuring absorbance at 680 nm every 1−2 days (see Supporting Information). For growth comparison with nutrient-limited conditions, nitrogen-deficient media was prepared containing 33% nitrogen compared to that of normal media.

Lipid Extraction. Cultures (500−mL) were harvested 6 days into stationary phase based on absorbance values. Cells were concentrated by centrifugation and lyophilization to remove water. Nonpolar lipids were extracted by sonication and solvent (chloroform/methanol and PBS buffer). The chloroform layer was isolated, concentrated by rotorary evaporation, and further dried under vacuum.[56,57] Changes in lipid levels for batch cultures were determined by crude lipid extract mass. Lipid extracts were further analyzed for the following: (1) TAG presence and purity by ^{1}H nuclear magnetic resonance (NMR)

spectroscopy (sample spectra in Supplementary Figure S10); (2) fatty acid composition using gas chromatography mass spectrometry (GC−MS) after TAG transesterification; and (3) composition of TAG using solid-phase extraction and analysis by matrix assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry. A comparison of spectra is provided in Supplementary Figures S11 and S12. By NMR spectroscopic analysis, no significant amount of compound or DMSO was observed in the lipid extract, verifying the confidence in the lipid extract mass.

■ ASSOCIATED CONTENT

Supporting Information

Complete list of materials and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: akfranz@ucdavis.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by the University of California Davis and Chevron Technology Ventures. A.K.F. acknowledges 3M for a Nontenured Faculty award. L.A.A. is supported by an NSF Graduate Research Fellowship and UC Davis Borge Fellowship. We thank P. Beal and J. Shaw at UC Davis for helpful discussion. D. Lam, A. Silva, T. Nguyen, and C. Pham are acknowledged for technical assistance related to microalgae growth and Nile Red analysis optimization studies.

■ REFERENCES

(1) Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., and Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J. 54*, 621−639.

(2) Wijffels, R. H., and Barbosa, M. J. (2010) An outlook on microalgal biofuels. *Science 329*, 796−799.

(3) Sheehan, J., Dunahay, T., Benemann, J., and Roessler, P. (1998) *A look back at the US Department of Energy's Aquatic Species program - Biodiesel from Algae*, p 328, National Renewable Energy Laboratory, Golden, CO.

(4) Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnol. Adv. 25*, 294−306.

(5) Suen, Y., Hubbard, J. S., Holzer, G., and Tornabene, T. G. (1987) Total lipid production of the green alga *Nannochloropsis* sp. qII under different nitrogen regimes. *J. Phycol. 23*, 289−296.

(6) Cerón Garcia, M. C., Sánchez Mirón, A., Fernández Sevilla, J. M., Molina Grima, E., and Garcia Camacho, F. (2005) Mixotrophic growth of the microalga *Phaeodactylum tricornutum*: Influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochem. 40*, 297−305.

(7) Kilian, O., Benemann, C. S. E., Niyogi, K. K., and Vick, B. (2011) High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proc. Natl. Acad. Sci. U.S.A. 108*, 21265−21269.

(8) Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y., and Richmond, A. (1987) Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. *Biomass 12*, 37−47.

(9) Yu, W.-L., Ansari, W., Schoepp, N., Hannon, M., Mayfield, S., and Burkart, M. (2011) Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. *Microb. Cell Fact. 10*, 91−102.

(10) Roesler, K., Shintani, D., Savage, L., Boddupalli, S., and Ohlrogge, J. (1997) Targeting of the *Arabidopsis* homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. *Plant Physiol. 113*, 75−81.

(11) Dehesh, K., Tai, H., Edwards, P., Byrne, J., and Jaworski, J. (2001) Overexpression of 3-ketoacyl-acyl-carrier protein synthase IIIs in plants reduces the rate of lipid synthesis. *Plant Physiol. 125*, 1103−1114.

(12) Vigeolas, H., and Geigenberger, P. (2004) Increased levels of glycerol-3-phosphate lead to a stimulation of flux into triacylglycerol synthesis after supplying glycerol to developing seeds of *Brassica napus L.* in planta. *Planta 219*, 827−835.

(13) Vigeolas, H., Waldeck, P., Zank, T., and Geigenberger, P. (2007) Increasing seed oil content in oil-seed rape (*Brassica napus L.*) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. *Plant Biotechnol. J. 5*, 431−441.

(14) Jain, R., Coffey, M., Lai, K., Kumar, A., and MacKenzie, S. (2000) Enhancement of seed oil content by expression of glycerol-3-phosphate acyltransferase genes. *Biochem. Soc. Trans. 28*, 958−961.

(15) Zou, J., Katavic, V., Giblin, E., Barton, D., MacKenzie, S., Keller, W., Hu, X., and Taylor, D. (1997) Modification of seed oil content and acyl composition in the *Brassicaceae* by expression of a yeast sn-2 acyltransferase gene. *Plant Cell 9*, 909−923.

(16) Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., and Goodenough, U. (2009) Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. *Eukaryotic Cell 8*, 1856−1868.

(17) Siaut, M., Cuine, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylides, C., Li-Beisson, Y., and Peltier, G. (2011) Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnol. 11*, 7−21.

(18) Li, Y., Han, D., Hu, G., Sommerfeld, M., and Hu, Q. (2010) Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. *Biotechnol. Bioeng. 107*, 258−268.

(19) Moellering, E. R., and Benning, C. (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. *Eukaryotic Cell 9*, 97−106.

(20) Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J. 58*, 165−174.

(21) Schreiber, S. L. (2005) Small molecules: the missing link in the central dogma. *Nat. Chem. Biol. 1*, 64−66.

(22) Walsh, D. P., and Chang, Y.-T. (2006) Chemical genetics. *Chem. Rev. 106*, 2476−2530.

(23) Stockwell, B. R. (2004) Exploring biology with small organic molecules. *Nature 432*, 846−854.

(24) Lemieux, G. A., Liu, J., Mayer, N., Bainton, R. J., Ashrafi, K., and Werb, Z. (2011) A whole-organism screen identifies new regulators of fat storage. *Nat. Chem. Biol. 7*, 206−213.

(25) Kaufman, C. K., White, R. M., and Zon, L. (2009) Chemical genetic screening in the zebrafish embryo. *Nat. Protoc. 4*, 1422−1432.

(26) Burns, A. R., Kwok, T. C. Y., Howard, A., Houston, E., Johanson, K., Chan, A., Cutler, S. R., McCourt, P., and Roy, P. J. (2006) High-throughput screening of small molecules for bioactivity and target identification in *Caenorhabditis elegans*. *Nat. Protoc. 1*, 1906−1914.

(27) Blackwell, H. E., and Zhao, Y. (2003) Chemical genetic approaches to plant biology. *Plant Physiol. 133*, 448−455.

(28) Maucourt, K., Agarwal, M., René, B., and Fermandjian, S. (2002) Use of *Chlamydomonas reinhardtii* mutants for anticancer drug screening. *Biochem. Pharmacol. 64*, 1125−1131.

(29) Avasthi, P., Marley, A., Lin, H., Gregori-Puigjane, E., Shoichet, B. K., von Zastrow, M., and Marshall, W. F. (2012) A chemical screen identifies Class A G-protein coupled receptors as regulators of cilia. *ACS Chem. Biol. 7*, 911−919.

(30) Chen, W., Zhang, C., Song, L., Sommerfeld, M., and Hu, Q. (2009) A high throughput Nile red method for quantitative

measurement of neutral lipids in microalgae. *J. Microbiol. Methods 77*, 41−47.
(31) Danielewicz, M. A., Anderson, L. A., and Franz, A. K. (2011) Triacylglycerol profiling of marine microalgae by mass spectrometry. *J. Lipid Res. 52*, 2101−2108.
(32) Knothe, G. (2009) Improving biodiesel fuel properties by modifying fatty ester composition. *Energy Environ. Sci. 2*, 759−766.
(33) White, D. A., Pagarette, A., Rooks, P., and Ali, S. T. (2012) The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures. *J. Appl. Phycol.*, 1−13.
(34) Gardner, R. D., Cooksey, K. E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R. P., Gerlach, R., Fields, M. W., and Peyton, B. M. (2012) Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte *Scenedesmus* sp. and the diatom *Phaeodactylum tricornutum*. *J. Appl. Phycol. 24*, 1311−1320.
(35) Mazur, H., Konop, A., and Synak, R. (2001) Indole-3-acetic acid in the culture medium of two axenic green microalgae. *J. Appl. Phycol 13*, 35−42.
(36) Piotrowska, A., Czerpak, R., Pietryczuk, A., Olesiewicz, A., and Wędołowska, M. (2008) The effect of indomethacin on the growth and metabolism of green alga *Chlorella vulgaris* Beijerinck. *Plant Growth Regul. 55*, 125−136.
(37) Bishop, D. G., and Smillie, R. M. (1970) The effect of chloramphenicol and cycloheximide on lipid synthesis during chloroplast development in *Euglena gracilis*. *Arch. Biochem. Biophys. 139*, 179−189.
(38) Schimmel, R. J. (1984) Stimulation of cAMP accumulation and lipolysis in hamster adipocytes with forskolin. *Am. J. Physiol. 246*, C63−C68.
(39) Jay, A. (1996) Effects of organic solvents and solvent-atrazine interactions on two algae, *Chlorella vulgaris* and *Selenastrum capricornutum*. *Arch. Environ. Contam. Toxicol. 31*, 84−90.
(40) Gurtovenko, A. A., and Anwar, J. (2007) Modulating the structure and properties of cell membranes: The molecular mechanism of action of dimethyl sulfoxide. *J. Phys. Chem. B 111*, 10453−10460.
(41) McNeil, S. D., Nuccio, M. L., and Hanson, A. D. (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. *Plant Physiol. 120*, 945−949.
(42) Sanmartin-Suarez, C., Soto-Otero, R., Sanchez-Sellero, I., and Mendez-Alvarez, E. (2011) Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. *J. Pharmacol. Toxicol. Methods 63*, 209−215.
(43) De La Vega, F. M., and Mendoza-Figueroa, T. (1991) Dimethyl sulfoxide enhances lipid synthesis and secretion by long-term cultures of adult rat hepatocytes. *Biochimie 73*, 621−624.
(44) Lee, P. A., and de Mora, S. J. (1999) Intracellular dimethylsulfoxide (DMSO) in unicellular marine algae: Speculations on its origin and possible biological role. *J. Phycol. 35*, 8−18.
(45) Sunda, W., Kieber, D. J., Kiene, R. P., and Huntsman, S. (2002) An antioxidant function for DMSP and DMS in marine algae. *Nature 418*, 317−320.
(46) Okumura, Y., Koyama, J., Takaku, H., and Satoh, H. (2001) Influence of organic solvents on the growth of marine microalgae. *Arch. Environ. Contam. Toxicol. 41*, 123−128.
(47) Wang, X., and Tian, W. (2001) Green tea epigallocatechin gallate: A natural inhibitor of fatty-acid synthase. *Biochem. Biophys. Res. Commun. 288*, 1200−1206.
(48) Kamiyama, O., Sanae, F., Ikeda, K., Higashi, Y., Minami, Y., Asano, N., Adachi, I., and Kato, A. (2010) In vitro inhibition of α-glucosidases and glycogen phosphorylase by catechin gallates in green tea. *Food Chem. 122*, 1061−1066.
(49) Saffari, Y., and Sadrzadeh, S. M. H. (2004) Green tea metabolite EGCG protects membranes against oxidative damage in vitro. *Life Sci. 74*, 1513−1518.
(50) Foyer, C. H., and Shigeoka, S. (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol. 155*, 93−100.
(51) Ledford, H. K., and Niyogi, K. K. (2005) Singlet oxygen and photo-oxidative stress management in plants and algae. *Plant Cell Environ. 28*, 1037−1045.
(52) Tanaka, S., Ikeda, K., Miyasaka, H., Shioi, Y., Suzuki, Y., Tamoi, M., Takeda, T., Shigeoka, S., Harada, K., and Hirata, K. (2011) Comparison of three *Chlamydomonas* strains which show distinctive oxidative stress tolerance. *J. Biosci. Bioeng. 112*, 462−468.
(53) Georgianna, D. R., and Mayfield, S. P. (2012) Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature 488*, 329−335.
(54) León-Bañares, R. (2004) Transgenic microalgae as green cell-factories. *Trends Biotechnol. 22*, 45−52.
(55) We have observed that increases in lipid levels based on Nile Red analysis are amplified in the initial microplate screening compared to effects observed using gravimetric analysis in batch cultures. While this may partially be attributed to the semi-quantitative nature of Nile Red analysis in high-throughput format when used for multiple algae strains, it may also be attributed to lower lipid recovery in batch cultures using standard lipid extraction conditions. See: Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem. 226*, 497−509. Recent investigations in our own lab have shown that a second extraction of buoyant high-lipid cells can lead to up to 10% increased recovery of lipids. Furthermore, Jones et al. have recently reported the use of 2-ethoxyethanol as a better solvent for lipid extraction that increases recovery up to 200% in *Chlorella* sp. See: Jones, J., Manning, S., Montoya, M., Keller, K., and Poenie, M. (2012) Extraction of Algal Lipids and Their Analysis by HPLC and Mass Spectrometry. *J. Am. Oil Chem. Soc. 89*, 1371−1381.
(56) Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem. 226*, 497−509.
(57) Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., and Oh, H.-M. (2010) Comparison of several methods for effective lipid extraction from microalgae. *Bioresour. Technol. 101*, S75−S77.