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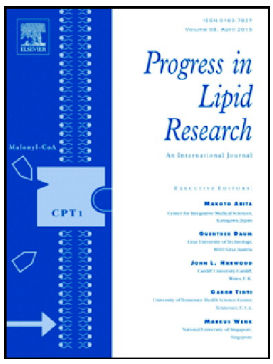

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Innovations in improving lipid production: Algal Chemical Genetics

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Abstract

Perturbation of a biological system with small molecules to achieve a desired phenotype or activity is commonly referred as chemical genetics. In pharmaceutical discovery, this approach is most often employed in target-based screening but in plants systems the focus is primarily on phenotypic selection for commercially relevant phenotype generation such as crop improvement or disease and pathogen resistance. Likewise, algae are considered feedstock organisms for viable and sustainable biofuels and other high value products with commercial applications. Algal triacylglycerol synthesis is therefore an important target for chemical genetics using high throughput technologies. In this review, efforts are directed towards summarizing our present understanding of the regulation of algal triacylglycerol biosynthesis, highlighting critical enzymes in lipid and carbon metabolism that may be manipulated to increase lipid metabolism in algae. These enzymes and pathways are targets for chemical genetics with the focus on selection of small molecules as tools to improve triacylglycerol storage. Using case studies, we summarize how chemical genetics is being used in plant and microalgal systems to address these critical problems.

Keywords: Triaclyglycerol, Chemical genetics, Microalgae, Target identification, High throughput screening, Biofuels

Abbreviations

TAG Triacylglycerol; HTS high throughput screening; G3P glycerol-3-phosphate; GPAT acyl-CoA:glycerol-3-phosphate acyltransferase; LPA lysophosphatidic acid; LPAAT acyl-CoA:lysophosphatidic acid acyltransferase; PA phosphatidic acid; PAP Phosphatidic acid phosphatase; DAG diacylglycerol; DGAT Diacylglycerol acyltransferase; PDAT Phospholipid: diacylglycerol acyltransferase; ATGL Adipose triglyceride lipase; HSL Hormone-sensitive lipase; MGL Monoacylglycerol lipase; KASIII 3-ketoacyl-acyl-carrier protein synthase III; PEPC1 Phosphoenolpyruvate carboxylase 1; AMP Adenosine monophosphate; IMP Inosine monophosphate; WRI1 Wrinkled 1; CETSA Cellular Thermal Shift Assay; DARTS Drug Affinity Responsive Target Stability assays; SPROX Stability of Proteins from Rates of Oxidation; iTRAQ Isobaric Tags for Relative and Absolute Quantification; TMT Tandem Mass Tag; RNAi RNA interference; CRISPR Clustered Regularly Interspaced Short Palindromic Repeats; cAMP Cyclic adenosine monophosphate; AMP Adenosine monophosphate; RO5 Lipinski's Rule of 5; IC50/EC50 Half-maximal Inhibitory/Effective concentration; GC-MS Gas chromatography-mass spectrometry; LC-MRM Liquid chromatography-Multiple Reaction Monitoring; LDs Lipid Droplets.

1 Introduction

Chemical genetics can be defined as the use of small molecules to disrupt metabolism resulting in a specific phenotype [1-3]. Most organisms employ intricate networks of metabolites that feed into larger metabolic networks as required for growth, development and maintenance. Perturbing the function of metabolic networks has increased our understanding of the layers of complexity and interactions required by living organisms. The use of small drug-like molecules to alter metabolism by interacting with macromolecules including proteins, DNA, carbohydrates and lipids represents a promising area of research to further define macromolecular interactions and relationships within and between metabolic networks [1, 2, 4, 5]. These small compounds are generally selected by high throughput screening for their ability to induce a specific phenotype such as lipid body accumulation.

The use of small molecules to disrupt metabolism has a rich history. The historical records of medicinal chemistry include numerous examples where plant compounds were used to treat disease and/or infections. One of the earliest examples of such compounds is acetylsalicylic acid in extracts of Willow bark, which was used to alleviate pain as early as 4,000 years ago. This compound was patented for use against pain, fever and inflammation as aspirin in 1899 by Bayer [6]. Quinine, a plant product from the bark of Cinchona tree was first isolated circa1820 and used extensively in the treatment of malaria and babesiosis [4]. The discovery of penicillin in 1928 is the earliest landmark in the era that included designing and repurposing small molecules to alter biological processes [5, 7, 8]. Natural and synthetic compound libraries have been routinely screened, for example, to identify pharmaceuticals, food preservatives and for manufacturing processes [9, 10]. The current field of chemical genetics has the potential to target specific pathways and has provided powerful tools in addition to classical genetics approaches. Many small molecules are attractive in targeting a specific pathway because they allow temporary perturbation of the system in a conditional and dose-dependent manner. The field of plant chemical genetics has been extensively reviewed [2, 5, 11-15] and dovetails well with classical genetic methods to provide insights into the characterization of protein function using traditional mating or breeding schemes. These strategies often require selective pressure to identify a phenotype of interest, which may then lead to the identification of the responsible gene(s) [16, 17]. Once the target gene is identified, molecular genetic engineering methods can be applied to determine the function(s) of a specific gene after directed deletion, mutation or altered expression (e.g., RNAi [18]) and assessment of the resultant phenotype (see refs. [19, 20]).

The promise of employing algae for industrial biotechnology applications is rooted in their genetic and biochemical diversity, which provides opportunities to discover novel small molecules with

industrial and health applications [21]. Most algae are photosynthetic organisms and can fix CO_2 into sugars that enter central metabolism for use as macromolecular building blocks making them, in principle, sustainable feedstock for a wide variety of biologicals. They offer significant advantages over yeast and bacterial hosts, which require the addition of organic sources of carbon (e.g., glucose, acetate). It is important to recognize that in establishing long-term sustainability for any type of industrial process using algae will require full life-cycle assessments [22].

Triacylglycerol (TAG) is an important storage lipid and provides an important role in energy homeostasis and carbon storage [23]. Algal lipids are considered a viable, sustainable source of biofuels and other hydrocarbons with commercial applications. However, the application of genetic engineering in microalgae to increase accumulation of TAGs coincident with growth has been met with limited success [24-27]. TAG biosynthesis in algae, like higher plants, is a complex process. The microalgae *Chlamydomonas reinhardtii* is estimated to have 113 genes involved in lipid metabolism based on sequence analysis [28] while the diatom *Phaeodactylum tricornutum* is estimated to have 106 genes [29]. By comparison, the estimate for *Arabidopsis* is over 600 genes and approximately 120 enzymatic reactions that contribute to acyl-lipid metabolism [30]. A complication and challenge in fully understanding lipid metabolic pathways in algae is that some enzymes are multifunctional and carry out two or more activities. There has been a general consensus that algal TAG synthesis occurs primarily in response to stress such as nutrient deprivation; the most common of which is nitrogen limitation. Since N is essential for growth, this affects overall productivity and, while there is an increase in the synthesis of TAG, this is accompanied by a decrease in biomass that must be overcome to make algal biofuels commercially viable [31, 32]. Systems biology studies have begun to unravel the metabolic changes that occur during N starvation. Of note, quantitative proteomic and transcriptomic analyses have shown N limitation leads to a reprogramming of metabolism with a shift of carbon flux into storage molecules such as starch and TAGs [32, 33]. To obviate the need for stress induction of lipid production, current advancements in molecular genetics approaches, systems biology analyses, and the use of drug-like small molecules together offer opportunities to engineer/induce algae to produce TAG production with minimal impacts on growth [25, 34-38].

This review will provide an overview of current chemical genetics approaches that impact TAG biosynthesis in algae and will discuss how these efforts differ from genetic engineering methods directed to increase TAG biosynthesis. Importantly, an overview will be provided of high throughput screening (HTS) methods, assay development, data analysis practices, and the software platforms available for such analyses. This will be followed by discussions of newly identified small molecules that induce enhanced TAG synthesis. Potential target identification strategies after HTS screening will be directed toward key case studies in which chemical genetics was employed

in algae and higher plants. Perspectives will be offered that address future trends, opportunities and bottlenecks in applying chemical genetic applications to algae for industrial applications.

1.1 Triacylglycerol biosynthesis in algae

Triacylglycerol is a neutral lipid consisting of a glycerol backbone esterified to 3 fatty acids and primarily functions in energy storage. TAGs have limited solubility under aqueous conditions, which allows for the storage of carbon without affecting overall metabolic flux [39]. Mechanistic insight into algal TAG metabolism is based on data from *Arabidopsis* but differs in that synthesis is primarily plastidic [40]. In *Chlamydomonas*, TAG synthesis is induced under stress conditions including nutrient limitation. When N is limiting, lipid droplets containing TAG are apparent as early as 6 hours after N removal and the droplets continue to expand in number and size until growth terminates [41, 42]. The inclusion of acetate in the culture media during nutrient deprivation further increases TAG accumulation presumably by changing the carbon nitrogen balance [43, 44]. Photosynthesis is intrinsically linked with TAG biosynthesis; when photosystem II is blocked by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) TAG synthesis is significantly compromised [45]. In addition to N limitation, P or S limitation, high light, salt and heat stress also result in TAG accumulation [42, 45-50]. Chemicals such as Brefeldin A, fenpropimorph, antioxidant agents, lipase inhibitors, lipoxygenase inhibitors, chemicals with benzylpiperazine and adamantine moieties significantly induces TAG accumulation [37, 38, 51-54]. Similarly, the MAP kinase inhibitors U0126 and IBMX also induce TAG accumulation in algae[55]. Recently metabolomics analysis was used to decipher the mechanism of lipid accumulation induced by treatment with the antioxidant butylated hydroxyanisole in *Crypthecodinium cohnii* [56].

In algae, TAG biosynthesis requires the same enzymatic steps as defined in higher plants [57]. The presumed complete set of genes in *Chlamydomonas* required for TAG biosynthesis has been annotated [28]. In algae, the Kennedy pathway is the primary metabolic pathway and is referred to as acyl-CoA-dependent TAG biosynthesis (Figure 1). Triacylglycerol is produced through the sequential addition of acyl chains to glycerol-3-phosphate (G3P) specific acyltransferases [28, 30]. The G3P is first acylated at *sn-1* position by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) to produce lysophosphatidic acid (LPA) followed by the second acylation at *sn-2* position by acyl-CoA: lysophosphatidic acid acyltransferase (LPAAT) resulting in phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) dephosphorylates PA to *sn*-1,2-diacylglycerol (DAG). Diacylglycerol acyltransferase (DGAT) catalyzes the last step of TAG synthesis through the addition of a third acyl chain to the *sn-3* position of DAG. An alternative route for TAG synthesis is an acyl CoA independent pathway and proceeds through phospholipid: diacylglycerol

acyltransferase (PDAT), which directly transfers acyl-CoA from phospholipid to DAG to produce TAG and a lysolipid [58-60]. *Chlamydomonas* has one gene encoding PDAT, which appears to have broad specificity for different phospholipids and galactolipids [58].

1.2 Genetic engineering to improve TAG biosynthesis

Over the past ten years, significant advances have been made in the ability to modify algal genomes to increase TAG accumulation through genetic engineering. These strategies are directed towards 5 main outcomes: [1] increasing the rate of acyl CoA and fatty acid and synthesis; [2] inhibition of lipases and ß-oxidation enzymes to inhibit degredation; [3] overexpression of Kennedy pathway enzymes involved in TAG synthesis; [4] manipulation of specific thioesterases and desaturases to control fatty acid chain length and saturation; and [5] over- or under-expression of specific transcription factors [25-27, 48, 59, 61-68]. These strategies have been used in different species of algae with some degree of success as summarized in Table 1.

The earliest efforts to increase TAG synthesis were directed towards the overexpression of native acetyl CoA carboxylase in the diatoms *Cyclotella cryptica* and *Navicula saprophila* were largely unsuccessful [69, 70]. Reducing the expression of CrPEPC1 using RNAi increased TAG levels by 20% [63]. Down-regulating both PEPC isoforms (CrPEPC1 and CrPEPC2) resulted in a further modest increase in TAG levels that was suggested to occur due to the flux of carbon away from the TCA cycle thereby increasing the availability of acetyl-CoA for fatty acid biosynthesis [71]. Likewise, reduction of citrate synthase (Phytozome ID: Cre03.g149100; CIS2) expression using RNAi increased TAG level by 169%. By contrast, overexpression of the same citrate synthase decreased TAG levels nearly two-fold [27]. Efforts directed to manipulate genes in the fatty acid and TAG biosynthesis pathways were variable. The overexpression of KAS2 increases the C18 fatty acids but with no changes in TAG levels [72]. The overexpression of C12 and C14 thioesterases did not increase TAG levels in the microalgae *Phaeodactylum tricornutum* [73].
In contrast to the manipulation of fatty acid biosynthesis enzymes, studies directed to change the expression of the TAG biosynthesis enzymes were moderately more successful. Overexpression of the type-2 diacylglycerol acyltransferase (DGAT2) in the marine diatom *P. tricornutum* resulted in a 35% increase in TAGs [62]. *Chlamydomonas* has 5 DGAT2 genes (CrDGAT2 1-5). Multiple protein sequence alignment showed significant differences but Pfam analysis has demonstrated each contains a diacylglycerol acyltransferase domain. Overexpression of CrDGAT2-1 and CrDGAT2-5 increases total lipid content 21% and 43% respectively [59]. When all the 5 genes of the Kennedy pathway including glycerol-3-phosphate dehydrogenase (G3PDH), GPAT, LPAAT, PAP and DGAT from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* were cloned into *Chlorella*

minutissima, there was a moderate two-fold increased accumulation of TAG [74]. When GPAT from the oleaginous green microalga *Lobosphaera incisa* (Trebouxiophyceae) was expressed in *Chlamydomonas* there was a 50% increased TAG levels [26]. Moreover, when DGTT1 from *Chlamydomonas* was overexpressed in *Scenedesmus obliquus* TAG levels increased 35% under N starvation [75]. Overexpression of PtDGAT2A in diatom *P. tricornutum* significantly increased TAG levels [62].

Apart from the genes encoding biosynthetic enzymes detailed above, other genes have been targeted with the goal of increasing TAG levels. Overexpression of the lipid body associated protein PNPLA3 in *P. tricornutum* increased TAG levels by 70% [76]. Likewise, overexpression of a NAD(H) kinase (a key source of cellular reductant NADH required in variety of abiotic stress responses) from *Arabidopsis* increased lipid content by 110% when expressed in the industrial oleaginous microalgae *C. pyrenoidosa* with no adverse effect on the growth [48]. When expression of AMP deaminase is reduced using RNAi, TAG levels increase by 25% [77]. Reduced expression of chrysolaminarin synthase in the diatom *Thalassiosira pseudonana* resulted in a more than 2-fold increase in TAG [78]. Likewise reducing the expression of UDP-glucose phosphorylase in *P. tricornutum* also increases TAG accumulation [79]. Triacylglycerol and starch represent the primary carbon sinks in algae. During the first 24 hours of N starvation there is a rapid accumulation in starch [42]. Not surprisingly, blocking starch synthesis in the "starchless" mutants of *Chlamydomonas* increase TAG accumulation under N starvation and indeed the starchless strains are amongst the highest lipid producing strains identified to date [24]. Essentially all of these methods function to shunt carbon from one compound class into storage lipids.

The majority of genes targeted for genetic manipulation were identified using molecular and biochemical studies. More recently, this has been expanded to include systems level studies (e.g., genomic, transcriptomic and proteomic analyses) of cells induced to produce lipids in response to stress [80]. Of particular interest has been the identification of transcription factors and regulators linked to the coordinated metabolic response that results in TAG accumulation [58, 81-83]. These transcription factors represent important targets because changes in the activity or abundance of one target may result in cascade effects on other metabolic genes in the same regulatory network. Studies in higher plants reported over-expression of a DOF-type transcription factor is correlated with increased TAG accumulation in seeds [84]. When this was tested in *Chlamydomonas* there was a two-fold increase in total lipids [85]. The overexpression of the AP2 type transcription factor *Wrinkled1* from *Arabidopsis* (AtWR1) increased TAG accumulation by 40% in *Nannochloropsis salina* [86]. Similar results were obtained through the overexpression of the bZIP transcription

factor in *N. salina* [68]. More recently, the abundance of a Zn(2)Cys(2) transcription factor was reduced in *N. salina* using a CRISPR-Cas9 genome editing platform that introduced an RNAi against the TF, which resulted in a two-fold increase lipid production without adverse effects on growth [25].

2 Applications of chemical genetics to probe biological function

The genetic studies detailed above, while highly valuable in the identification of targets for increased TAG synthesis, have only been marginally successful in unlinking TAG accumulation from decreased growth and thus biomass. To make algae viable as a feedstock for biofuel production, it is essential to develop robust strategies to increase TAG accumulation without compromising growth and biomass yields. As an alternative to nutrient starvation the application of chemical genetics to identify lipid storage inducers in algae is highly promising. Among the first reported study, Franz and colleagues screened a small compound library to identify chemicals that induced TAG accumulation in 4 different marine algae (*Nannochloropsis salina*, *N. oculata*, *Nannochloropsis* sp. and *Phaeodactylum tricornutum*) without nutrient deprivation or compromising growth [37]. Among the hits were the fungicide fenpropimorph , U0126 (a MAP kinase inhibitor), IBMX (a modulator of cAMP signaling), and Brefeldin A, which rapidly induces TAG accumulation in *C. reinhardtii* CC125 [51] [52, 53, 55] [54].

Our recent work has taken advantage of a high throughput screening system developed to select small molecule chemical inducers of lipid storage in *C reinhardtii* [38]. From over 43,000 compounds screened, 243 compounds were identified that clustered into 5 distinct structural scaffolds. These compounds were effective at low dosage, stimulated lipid production during growth and were effective in multiple algal species including *Chlamydomonas, Chlorella sorokiniana* UTEX 1230, *C. vulgaris* UTEX 395, *Tetrachlorella alterens* UTEX 2453 [38]. This demonstrates the potential and strength of high throughput screening approaches in algae.

2.1 Rationale for applying chemical genetics

There are a number of parallels between chemical genetics in traditional genetic studies. A chemical that inhibits protein function or expression is similar to a loss-of-function phenotype developed using molecular genetic tools. Likewise, chemicals that are agonists of protein function are similar to gain-of-function and over-expression approaches. Apart from the loss -/gain-of-function, chemical genetic approaches have additional key advantages over traditional genetic methods. For example, a chemical can interfere with more than one isoform of a protein as opposed to targeting a single protein using traditional genetics. If reversible, these small molecule chemicals can be added and removed providing temporal control to perturb protein function, a feature that circumvents lethality due to mutation. Also, by applying chemical genetic methods, small

molecules can be added in dose-dependent manner, which allows fine-tuning to modify a phenotype, which is more difficult to achieve through traditional genetic methods. Importantly, genetic engineering requires prior knowledge of the function of a given gene and this is not essential when applying chemical genetic methods.

Chemical genetics can be applied in both forward and reverse directions. As illustrated in Figure 2, forward chemical screening is useful for identifying small molecules that induce a phenotype of interest without knowledge of the target while in reverse chemical screening a single protein is targeted with a chemical activator or inhibitor. Regardless of the approach, the small molecules used in chemical genetics have the capacity to induce a biological effect conditionally and in dose-dependent manner, avoiding variable and possibly unrelated impacts that may occur using a more standard genetics approach. Further, the small molecules can be introduced in a conditional manner, may be added at any time and at variable concentrations over the course of an experiment. Moreover, depending on their size and physiochemical properties, these small molecules may penetrate the cell to reach their target or may interact with cell surface receptors, much like drugs and some hormones. Many genetic approaches do not lead to the expected phenotype due to compensatory mechanisms, such as multiple isoforms of the same protein, whereas small molecule treatments may obviate this by interacting with a site common to all or most isoforms. These small molecule effectors are tunable by varying their concentrations. In the case of algae, another potential obstacle using these approaches is the barrier imposed by the pectin-cellulosic cell but this is virtually ignored in most phenotypic screening plans when considering the chemical library. To date, the application of chemical genetic methods is more common in animal models and cell-based systems, with only a few conducted in higher plants or algae. There are several reviews addressing chemical genetics in plants [4, 5, 14, 87-91] and algae [37, 38, 51, 54, 92] that the reader may refer to for additional information.

2.2 Selection of small molecule libraries and concentrations for screening

Chemical genetic approaches rely on carefully designed chemical libraries of thousands of structurally diverse compounds and unique targeted assay systems [89]. Most commercial libraries are designed to obey Lipinski's rules for solubility and permeability in mammalian cells as desired for drugs [1, 93]. The Lipinski Rule of 5 (RO5) describes the physicochemical properties of molecules for orally administrated drugs that would likely be absorbed into cells [94, 95]. The most common physiochemical properties include molecular mass < 500 daltons; log P (octane-water partition coefficient) < 5; number of H bond donors < 5; H bond acceptors < 10. The RO5 does not predict whether or not the compound would be active against a specific target, but violation of any

of these parameters makes the molecule less suitable as a drug. There are exceptions to RO5 such as herbicides, insecticides and natural products [96]. In the case of agricultural applications, spraying is the preferred method of delivery. The barrier for uptake in plants is different than that for more traditional pharmaceutical applications, which includes the leaf cuticle, root or shoot uptake or uptake via xylem and phloem and in case of algae, presence of thick pectin-cellulose cell wall and polysaccharide sheath. Moreover, the dosage frequency can be less as compared to animal models. Thus, Lipinski's RO5 may not be as useful in the case of plants or algae.

Several commercial libraries are available that generally follow RO5 [97]. The chemical libraries usually differ in composition, which will affect the outcome of the screening experiment as discussed in detail elsewhere [97]. The bioactive collections contain compounds with well-defined biological activities. These libraries are generally smaller in size than undefined collections. A selected list of chemical screening library collection is provided as Table 2.

The selection of the concentration of compounds to be used in a given screening method is somewhat empirical. Generally, chemical libraries are provided either dried or as a 10 mM starting stock in DMSO. As a general rule, initial screening is performed at a fixed concentration ranging from about 1-10 µM. This also depends on whether the route of screening applies the forward or reverse approach (e.g. *in vivo* cell-based assays versus a specific purified enzymatic target). In target-based reverse screening carried out *in vitro*, the compounds generally have higher apparent activity compared with cell-based assays since they have direct access to the binding pocket of the target without any cell membrane and/or cell wall restrictions, and thus lower screening concentrations are preferable (generally in the nanomolar range). In a typical forward *in vivo* phenotypic screen of an organism or cell line, the small molecules have to cross the cell barrier and/or intracellular membranes and the target is assumed to be in very low concentration, hence, requiring higher effective concentrations. A recent review reported chemical screening in plant systems may employ concentrations as high as 20-50 µM [98].

3 Development of high throughput screening (HTS) platforms

Assay development is an important step in high throughput screening. The high throughput screening assay must be reliable, robust and reproducible. It is also important to consider whether or not the phenotype to be scored is suitable using a microplate format. The basic assay read-out often consists of a quantitative measurement using absorbance/fluorescence or luminescence reporters. The nature of the response should be clearly defined in terms of the phenotype of interest such as increased or decreased signal. HTS assays fall broadly into three general categories: [1] purified protein/enzyme assays; [2] cell-free lysate assays; and [2] cell- or organism-based phenotypic assays. Purified enzymes/protein assays are typical examples of reverse screening

approaches while cell extracts and phenotypic assays using whole cells are examples of forward chemical screening. In the case of phenotypic screening, optimization of cell density is critical. Higher cell densities require higher initial concentrations of the library compounds; conversely, if the cell density is too low, then the concentration of the compound has the risk of being toxic to the cells. Hence, assay optimization is a critical step before the actual screening. Another consideration in assay development is to test that the phenotype is not influenced by the vehicle solvent (e.g. DMSO or ethanol). A good initial estimate is that the solvent vehicle should not exceed 1% of the total volume of the cell suspension per well. The assay should be clearly defined to include positive and negative controls to assist in measuring the assays dynamic range. Positive controls are conditions that would produce a similar phenotype as that desired of an active compound. Negative controls are generally cells that are treated with "empty vehicle" (for example, DMSO) demonstrated to have no activity in the assay. For example, N starved algal cells produce a hyper-lipid accumulating phenotype and this phenotype can be detected by adding the lipophilic dye Nile red or a fluorescent dye BODIPY. Hence, the increased fluorescence Nile Red or BODIPY signal from cells starved for nitrogen would provide a reasonable positive control for lipid accumulation. The negative control is usually cells treated with the vehicle alone. Additional examples of assay development in algae have been described previously [37, 54, 92].

3.1 Statistical data analysis of the high-throughput screening

High-throughput screening is a complex large-scale process where thousands of compounds are screened in order to identify potential drug-like molecules. During a primary screen, it is a general practice to perform one replicate due to reagent and cost considerations. Therefore, care must be taken to ensure the assay is specific, robust, and offers a relatively wide dynamic range of sensitivity between positive and negative controls [98]. Depending upon the number of compounds to be screened and the availability of the biological materials, microtiter plates with 96-, 384- or 1536- wells are employed. In plant HTS efforts, such as growing single seedlings, large well microplates (24 or 48-well plates) are suitable but this may limit the size of the screening library to between 500-10,000 compounds while in algal screens low volume 96-384-well microplates is the preferred choice. Once the format is established, the quality control (QC) parameters must be established. Reference controls are placed within each plate to determine plate-to-plate variation and to estimate effectiveness of the screen. For commercial libraries, the compound collections are usually organized so that the first and last columns on the microtiter plates are empty and reserved for the controls. Adding both positive and negative controls on the same plate helps to identify the hits with a higher degree of confidence (Figure 3). Moreover, in reporter-based or dye-based screening assays, the interaction of the chemical compound with the dye/reporter may result in false

positive or false negative results for some compounds. For example, typically as many as 2-3% of the chemical library interferes with the luminescent luciferase activity assessment [99]. Therefore, it is important to verify activity by testing the compound alone using the same wavelength to establish that the compounds do not contribute fluorescence or absorbance. Several pre-processing and statistical methods are available to identify the range of compound potencies and efficacies from the HTS screen, quality control and robust identification of the "hit" molecules (detailed in Box 1).

3.2 Software platforms for HTS and chemoinformatics analysis

HTS data analysis is complex and can be time-intensive. The number of data points generated in these experiments is high and these must be processed efficiently to identify biologically relevant signals. The HTS data may suffer from signal variation caused by batch, plate, and/or instrumental handling [100]. The identification of putative "hits", i.e., compounds imparting the desired phenotype, is highly dependent on choosing the correct data processing routine. Several tools are available that offer straightforward analysis and correct for plate-to-plate variation and systematic errors. A short compilation of commercial as well as open source software tools is listed in Table 3.

3.3 Verification of hits from the primary screen and establishment of efficacy

Once primary hits are identified, the next critical step is to rigorously verify the compound's efficacy. To do this, the screening assay is repeated on the potential hits identified from the primary screen to establish robust and reliable dose-response activities. An important parameter to evaluate the chemical's efficacy is to establish the half-maximum inhibitory/effective concentration (IC_{50}/EC_{50}). To establish the accurate IC_{50}/ EC_{50} concentration for a given compound, it is essential to include a range of compound concentrations to determine both the minimum and maximum effective dosages. Establishment of IC_{50}/ EC_{50} will help to avoid deleterious effects of high concentrations and to minimize false positives and negatives. The next step for validation is to perform orthogonal independent assays to confirm the biological activity of the selected "hits". The orthogonal assay might include, as examples, a different reporter, determination of endogenous gene expression or other methods that verify the biological effect addressed in primary screen. This is also essential to uncover off-target effects. For example, in our previous work, a large library was screened for chemical inducers of lipid accumulation initially using Nile Red lipophilic dye to select active compounds. Of 367 compounds identified as hits in the primary screening, only 243 were confirmed by rescreening and dose-response analysis [38]. To verify the TAG inducing phenotype for a subset of lead hits, cells were treated with compound under the conditions of the primary screening and then lipid accumulation was verified by total fatty acid identification and quantification using GC-MS, targeted quantification of complex lipids using LC-MRM, and

confocal microscopy to visualize lipid droplets. These secondary screens unequivocally established the desired biological activity of the final set of selected lead compounds.

Defining both the selectivity and specificity of a given compound for the target is essential. Ideally each active compound should have only one target (e.g., enzyme activity) thereby minimizing off target and side effects [14, 98, 101]. Compounds that are destined for use as drugs or in food sources must pass food and drug administration tests for safety and efficacy (https://www.fda.gov/default.htm). Likewise, for compounds that may be deployed in the environment or deposited in environmental waste, Environmental Protection Agency guidelines must be followed (https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances). Strategies to increase algal oil production for biofuels fall under the latter guidelines. To establish the selectivity of a compound, its impact on various biological processes needs to be evaluated. Common among these is toxicity and metabolism of the primary compounds as well as metabolites and degradation products. For example, Wase et al. tested selected activators of TAG synthesis in algae for various biochemical readouts including changes in growth, biomass yield, starch production, fatty acid levels, effects on photosynthetic pigments and efficacy in multiple algal species [38]. Basic physiological processes such as photosynthetic and respiration rates as well as markers of stress, such as levels of reactive oxygen species and glutathione levels, were also evaluated for cells treated with lipid storage inducing compounds (unpublished data).

4 Small molecules to target lipid metabolism leading to enhanced TAG synthesis

Lipids are the primary components of membranes that are essential to cellular organization, function as storage compounds for energy, and may have bioactive roles that contribute to cellular regulation. Lipid metabolic pathways are dynamic and include fatty acid synthesis and degradation, complex lipid synthesis of membrane and neutral lipids including TAGs, and the synthesis of bioactive lipids, many of which are involved in cell signaling. The application of chemical biology is ideal to target lipid metabolism with the goal of increasing TAG accumulation. This requires a full understanding of the lipid metabolic networks and enzymes and how they can be manipulated such that lipid metabolic flux result in increased TAG synthesis (see Section 1.1 above). The identification of small molecules from the HTS strategies detailed above would be expected to modulate the function of key enzymes and regulatory molecules and processes to provide important tools to decipher biological function. Additionally, the compounds provide tools to understand how modulation of metabolic pathways and enzymes may lead to the desired phenotype, in this case the accumulation of TAG. As detailed below, a number of small molecules have been identified and effectively used to target increased TAG synthesis circumventing the need for genetic engineering methods.

4.1 Chemical inhibition of lipases

Lipases catalyze the hydrolysis of fatty acids from complex lipids. In algae, lipase inhibition has been suggested as one target to increase TAG accumulation. There are structural similarities between pancreatic lipase, lipoprotein lipase (LPL), hepatic lipase (LIPC) and endothelial lipase (LIPG) that have been exploited to develop inhibitors of this class of enzymes. The LPL and LIPC both prefer TAGs as substrates, while LIPG prefers phospholipids [102]. Sulfonylfuran urea 2 is a potent inhibitor of LPL (IC_{50} = 0.1μM) and causes marked elevation of plasma TG levels [103]. Although undesirable in humans, the activity of this compound could be beneficial for algal biofuel research to inhibit related algal lipases. Several compounds showed excellent inhibitory activity towards LIPG and LIPC such as benzothiazoles showing an IC_{50} < 20 nM [104]. Specific lipases catalyze the degradation of lipid droplets (LDs) such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL). The ATGL catalyzes the initial step in TAG degradation and produces DAGs. Atglistatin is a competitive inhibitor of ATGL and can be effectively used for discouraging the degradation of LDs in algae [105]. HSL is a multifunctional enzyme with 10-fold activity with DAGs as substrates, compared with TAGs. Several pharmaceutical companies have developed HSL inhibitors based on carbomoyltriazole and carbamate [106, 107]. Several of these lipase inhibitors have been used to increase TAG accumulation in *Nannochloropsis* sp. Of note, it was reported that the pancreatic lipase inhibitor Orlistat (40 nM f.c.) increased Nile red fluorescence by 72% suggesting significant TAG accumulation [37]. Monoacylglycerol lipase (MGL) is an enzyme responsible for the last catabolic process in TAG degradation and generates glycerol as a by-product. A piperidine carbamate compound JZL184 is a highly selective inhibitor of MGL. In a recent study by Franz et. al., JZL184 when used at nM concentrations increased the apparent accumulation of neutral lipids in 4 marine algae species [37]. This study is discussed in detail as a case study (see below, section 6.4).

4.2 Chemicals that modulate the activity of acyltransferases

Acyltransferases catalyze acyl-CoA dependent ester bond formation between a fatty acid and an acceptor molecule such as glycerol. These enzymes play key role in the synthesis and remodelling of complex lipids including phospholipids and TAGs [108]. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first step of glycerolipid biosynthesis. At least two GPATs are predicted in *C. reinhardtii,* which are suggested targets for inhibition to lead to lipid accumulation. Support for this approach is evidenced by the observation that the expression of GPAT (Cre02.g143000.t1.2; PLSB1) is decreased significantly after nitrogen starvation for 4 hours commensurate with an increase in TAG synthesis and accumulation [58, 81]. FSG67 is an inhibitor of GPAT that inhibits all GPAT isozymes in mice [109]. The 1-acyl-sn-glycerol-3-phosphate (AGPAT) catalyzes acylation of lysophospholipids with acyl-CoA and produce phosphatidate. There are at least 5

predicted AGPATs present in *C. reinhardtii*. A non-competitive inhibitor of AGPAT, CT32228, has been described recently [110] and could potentially be used for defining the role of AGPATs in TAG biosynthesis in algae although this is not desirable for biofuel production purpose. The diacylglycerol acyltransferases (DGATs) catalyze the biosynthesis of TAGs by acylation of DAGs via the acyl-CoA-dependent pathway. Several inhibitors of DGATs are marketed by pharmaceutical companies [111] such as PF-04620110 (Pfizer), AZD7687 (Astra-Zeneca) (in Phase I trials), Pradigastat (Novartis) [112] and JNJ-DGAT2A and JNJ-DGAT2B (Johnson & Johnson). While, it is not desirable to inhibit AGPATs and DGATs for biofuel production, these inhibitors may be useful to define the roles of these enzymes in various metabolic routes leading to TAG biosynthesis. No activators of acyltransferases or other TAG biosynthetic enzymes have been described. It is possible that such compounds may be identified through screening for TAG accumulation in algae.

4.3 Other targets for improving oil production

Understanding microalgal lipid metabolism is critical for the improvement in the production of biodiesel. In recent years, many genes involved in lipid metabolism have been targeted for deletion or overexpression in an effort to improve lipid accumulation in higher plants such as *Arabidopsis*, rapeseed (*Brassica napus*), and soybean (*Glycin max*) to increase production of TAGs in seeds [73]. It has been proposed that increased fatty acid supply would increase oil synthesis and yield but these approaches have resulted in limited success [57]. Overexpression of acetyl-CoA carboxylase (ACCase) in seeds of *B. napus* yielded no significant change in seed lipid content but the same approach yielded a 5-fold increase in potato (*Solanum tuberosum*) [113]. Overexpression of 3-ketoacyl-acyl-carrier protein synthase III (KASIII) did not increase lipids in *A. thaliana* and *B. napus* [65], whereas overexpression of glycerol-3-phosphate dehydrogenase (G3PDH) resulted in a 40% increased lipid in the seeds of *B. napus* [114]. G3PDH catalyzes the synthesis of glycerol-3-phosphate which is a precursor for TAG biosynthesis. The compound MEDICA 16 (sc-203131; Santa Cruz Biotechnology) is an inducer of malic enzyme and mitochondrial G3PDH. It causes hypolipidemia in animal models, disfavoring fatty acid synthesis and favouring fatty acid oxidation [115]. Again, this compound may be of value in studying lipid metabolism in plants and algae but not in the production of lipids for commercial purposes. Recently, knockdown of phosphoenolpyruvate carboxylase 1 (PEPC1) an enzyme of the glycolytic pathway was shown to increase TAG levels 28-46% [71]. PEPC has an important role in carbon fixation and regulation of flux through TCA cycle. Previous studies showed that there is negative correlation between PEPC and lipid accumulation[64]. Using virtual chemical screening of 12, 918 publicly available compounds, potential small molecules inhibitor of PEPC were identified. To test the compounds,

recombinant PEPC from a angiospermic plant *Flaveria* (family: *Asteraceae*) was heterologously expressed in *E. coli* (BL21) and PEPC activity was monitored. Two compounds (+)-catechin and a quinoxaline compound AG 1433 inhibited activity and were shown to bind to an allosteric feedback inhibitor site [116].

Deletion of GUT2 encoding G3PDH in the yeast *Yarrowia lipolytica* resulted in a 3-fold increase in TAG. In this case, it was suggested that lipids were mobilized at the exponential phase of growth due to an increase in ß-oxidation of fatty acids. *Y. lipolytica* contains 6 acyl-CoA oxidases (AOX) catalyzing the rate-limiting step of peroxisomal beta-oxidation. Deletion of all 6 AOX genes in a Δ*gut2* mutant increased lipid 4-fold. Thus, inhibition of G3PDH along with AOX might be considered as another strategy for improving TAG accumulation. Recently a small-molecule screening study identified inhibitors of G3PDH [117]. The structure-activity analysis identified a core scaffold structure of benzimidazole-phenyl-succinamide essential for the inhibition of G3PDH. Modulation of the benzimidazole ring system improved the potency and reduced off-targets. Two compounds, iGP-1 and iGP-5, were identified as potent inhibitors of G3PDH and may have properties useful to increase algal lipid yield [118]. Treatment with trimetazidine, a 3-ketoacyl-CoA thiolase inhibitor, leads to a shift in metabolism of obese mice accompanied by accumulation of long-chain acyl-CoA and increases in TAG content [119]. Amiodarone inhibits mitochondrial beta-oxidation of fatty acids and twenty-four-hour dosage of this compound was shown to increase hepatic triglycerides by 150% in mice [120]. Therefore, use of either compound may inhibit fatty acid ß-oxidation to increase TAG accumulation.

Another possible route for increasing lipid production is to block energy-rich starch production. Previous work showed that disruption of ADP-glucose phosphorylase or isoamylase produced higher levels of TAGs during N starvation in *Chlamydomonas* [121]. Other commercially available compounds that inhibit starch accumulation and which might induce lipid accumulation include a urea-derived compound that targets glycogen phosphorylase (catalogue # sc-203975; Santa-cruz Biotechnology) [122] and deoxynojirimycin (catalogue # sc-201369; Santa-cruz Biotechnology), an inhibitor of beta-glucosidase and glucoamylase [123] c.

4.4 Targets for channelling acetyl-CoA to FA biosynthesis

All microorganisms are capable of synthesizing lipids but only the oleaginous species accumulate significant quantities (> 30% on dry cell basis). This has been suggested to be due to the production of high levels of acetyl-CoA, the basic building block of fatty acids [124, 125]. Pyruvate is the product of glycolysis and acetyl-CoA is produced from pyruvate by the activity of pyruvate dehydrogenase. The acetyl-CoA thus formed enters the TCA cycle to yield citrate. When in high levels, citrate is transported into the cytoplasm where it is reconverted to acetyl-CoA by the activity

of isocitrate lyase thus providing substrates for *de novo* lipid biosynthesis [125-127]. High levels of ATP and citrate generally favor fatty acid and lipid synthesis for storage purposes. However, in N deprivation in algae, lipid accumulation is associated with low energy levels and low AMP levels. This is suggested to occur by an increase in the expression and activity of AMP-deaminase to produce IMP (inosine monophosphate) and ammonia [77]. It was suggested that the excessive and abrupt decrease in AMP levels alters activity of the TCA cycle and NAD+/NADP+ isocitrate dehydrogenase (IDH) activity resulting in citrate accumulation in mitochondria [128]. When the citrate levels rise, excess citrate is transported to the cytosol by citrate:malate transporter [128] and is then cleaved to acetyl-CoA and oxaloacetic acid by the action of ATP-citrate lyase [129]. This suggests that inhibition of the activity of AMP-deaminase could be an important signal to switch the organism's metabolism to increase lipid biosynthesis. A recent report suggested that metformin, a widely prescribed medicine for type-II diabetes, activates the regulatory AMP kinase, at least in part, through inhibition of AMP deaminase [130]. Park et al. showed using time course transcript profiling in *Chlamydomonas* that during N starvation that RNA expression levels of AMP deaminase increase successively as N starvation progresses concomitant with a decrease in adenosine-5-monophosphate levels [41]. This observation points to the importance of AMP levels in regulating metabolism during N starvation. It is possible a similar alteration in AMP levels occurs during treatment with the lipid inducing compounds our laboratory has identified by high throughput screening. In this case, the levels of adenosine and guanosine in *Chlamydomonas* cells were significantly lower than non-treated controls. Reduction in the levels of adenosine and guanosine parallels the exhaustion of AMP in the cytosol. While AMP was not measured in those studies, it was noted that the levels of 2-Deoxyinosine 5-monophosphate were elevated after treatment with any of the 5 lipid inducing compounds [38]. Thus, reduction of AMP may be a common link for induction of lipid production. Treatment with the novel compounds also increased accumulation of isocitric acid, suggesting an important role for NAD+/NADP+ isocitrate dehydrogenase during the lipid inducing phase. Hence targeting the isocitrate dehydrogenase may also be an excellent strategy for induction of lipid production in algae. Several inhibitors of IDH such as Vorasidenib, IDH-305, Ivosidenib and GSK864 are available from commercial suppliers.

4.5 Small molecules for targeting transcription factors

One approach to influence metabolism globally is to regulate the activities of transcription factors (TFs) that control expression of genes in targeted pathways. However, while algal genome sequence analysis has led to the identification of putative transcription factors and other regulatory proteins, few studies have provided sufficient detail to understand regulatory networks. There have been TRs identified whose expression correlate with lipid accumulation that should be examined to determine

if there is a regulatory relationship (Table 1). Of interest, is the case of wrinkled 1 (WRI1) whereby overexpression resulted in 36-44% increase in TAG levels [86]. Similar results were obtained when a DOF-type transcription factor and selected bZIP transcription factors were overexpressed [68, 85]. Both DOF-type and bZIP transcription factors were known as stress regulators associated with lipid metabolism [68, 85]. Knock down of a Zn2Cys2 transcription factor using CRISPR-Cas9/RNAi system in *Nannochloropsis gaditana* yielded 2-fold enhancement of lipid accumulation in non-nitrogen starved cells [25]. These results provide some support for the notion that small compound activators or inhibitors of TFs may be selected and employed to increase lipid accumulation and storage. No chemicals thus far have been identified that function as TF acitvators, however a few chemicals have been identified as inhibitors but whether they would work in algae is a matter of investigation (https://www.scbt.com/scbt/browse/Transcription-Factor-Inhibitors/_/N-jjd14j). Recently, the expression of a bZIP TF was shown to be positively correlated with increased DGTT1 expression and increased TAG levels [83]. However, unpublished data from our laboratory indicated several transcription factors of bZIP family are significantly down-regulated when *Chlamydomonas* cells were treated with lipid inducing compounds (Compound WD30030 and WD10784; Wase et al unpublished). Activator protein 1 (AP-1; UniProt ID: P0512) has high sequence similarity with the bZIP family of transcription factors of *Chlamydomonas* (Score 111; E-value 2e-07). Compound SP 100030 (PubChem ID: 9910975) is a potent inhibitor of AP-1 transcriptional activity (IC_{50} = 50 nM) [131] and has been effectively used to reduce bZIP mediated transcriptional activity. In a recent transcriptome profiling of 3 *Nannochloropsis* species, the expression of several TFs including bZIP, NF-YC, C3H, AP2 and MYB were found to be highly correlated with lipid biosynthesis pathway enzymes, suggesting possible interrelated regulatory circuits. Two inhibitors of Myb were recently identified using a fluorescence-based chemical screening approach of natural sesquiterpene lactones (STLs) from a focused panel of 30 different STLs[132]. Among these, Mexicanin-I, an STL isolated from *Helenium mexicanum* was found to be a potent inhibitor of Myb-activity that functions in a dose-dependent manner [132-134]. Whether or not these inhibitors would work in algae to induce TAG accumulation is a matter for future investigation, nonetheless, this approach represents an attractive target to increase TAG production. These and additional metabolic targets, as well as small compound modulators of their activities, are presented in Figure 4

5 Target identification strategies

Once a small molecule is identified via phenotypic screening and the activity is confirmed, the next step required is the identification of the molecular target. This is necessary to fully unravel the effects of the compound on metabolism and to understand the mechanism of action. However, target identification can be hampered by the following limitations: 1) weak interaction between the

compound and the protein target caused by low-affinity binding; 2) low abundance of the potential target; 3) high abundance of non-specific binding proteins as may occur in cell plasma or sap [135] such as phycobilisomes anchored to thylakoid membranes as in the case of cyanobacteria *Nostoc punctiforme* [136]; and 4) lack of a suitable functional group to attach a tag on the small molecule that would prevent chemical modification for use as an affinity ligand. Few methods are available to reduce high abundance interfering proteins. Some commercially available kits include ProteoPrep 20 plasma immunodepletion kit (Sigma-Aldrich); affinity columns such as Multiple Affinity Removal System (MARS) from Agilent; or the liquid gel-free Free Flow Electrophoresis technique used previously for separation of high abundance phycobiliproteins [136].

The most common and direct method for target identification is to label the small molecule of interest, allow binding, followed by *in vitro* biochemical purification [137, 138]. Although this method is accurate, it is quite cumbersome and has limitations such as non-covalent binding between the ligand and the target, which may result in dissociation in the process of purification. Other approaches such as photoaffinity cross-linking, Surface-plasma resonance assay (SPR) or radiolabeling require knowledge of structure-activity relationship (SAR) and presence of suitable functional group as affinity chemistry is limited. To overcome these problems, alternative techniques have been described such as the Cellular Thermal Shift Assay (CETSA), Drug Affinity Responsive Target Stability Assays (DARTS), Limited Proteolysis coupled Selected reaction Monitoring (LiP-SRM) and Stability of Proteins from Rates of Oxidation(SPROX) [139-141]. These techniques are label-free and no chemical modification is required for designing functional chemical probes. An example of a successful target identification was carried out using DARTS while studying the effect of grape seed extract (GSE) in human colorectal cancer (CRC) cell lines. In this case, GSE causes ER stress and was shown to inhibit the PI3k-Akt-mTOR pathway specifically [142].

5.1 Target identification by the Cellular Thermal Shift Assay (CETSA)

The Thermal Shift Assay (TSA) has been used historically in drug discovery for target identification but this application is limited to purified native or recombinant proteins [143]. Recently, a modified method called the cellular thermal shift assay was described to assess small-molecule target engagement within the cell. This method takes advantage of drug-induced stabilization of the target protein under various experimental conditions [139]. Briefly, in a CETSA workflow, live cells, cell lysates or tissue samples are treated with either a vehicle control (e.g. DMSO) or the small molecule (Figure 5). The samples are then distributed in small aliquots and heated in a multi-temperature thermal cycler at different temperatures ranging from 37 ℃ to 60-65 ℃. If the compound-target complex is formed, the T_m of the drug-bound complex is generally

higher than that of the native protein from the vehicle-treated lysate sample. With increasing temperature, proteins start to unfold and aggregate at the bottom of the tube. The remaining soluble cellular proteins are separated from the aggregated proteins by centrifugation. Subsequently, the proteins that are complexed with the compound can be identified using MS-based approaches. Using this method, once the potential temperature at which the target-engagement is identified, then target-engagement potency can be measured using Isothermal-Dose Response Fingerprinting ($ITDRF_{CETSA}$). For ITDRF, the target engagement temperature is kept constant and the drug is added at varying concentrations to observe a thermal dose-response profile. A general schematic for a CETSA experiment is presented in Figure 5. To date, CETSA has been successfully used in several studies for target engagement using live cells as well as cell lysate [139, 144, 145]. An example that used CETSA is a study that confirmed a small molecule identified via a high-throughput screening is an inhibitor targeting nicotinamide phosphoribosyltransferase [145]. CETSA has also recently been successfully used in combination with multiplex quantitative proteomics to determine the thermal profile of more than 7000 proteins in human cells by mass spectrometry in samples treated with the kinase inhibitor staurosporin with over 50 proteins identified as potential targets [146]. The CETSA approach was also extended to membrane proteins of K562 cells employing different detergents including SDS, NP-40, CHAPS, CHAPSO, DDM, beta-octylglucoside, and Brij 35. Of these, 0.4% of NP-40 was effectively used for target identification [144]. Despite all these apparent advantages, CETSA has some potential drawbacks. For example, multi-domain proteins might not be identified if the chemical does not have access to the binding pocket. Moreover, in plant-based studies, there are relatively few proteins for which antibodies are available for western blot based detection. This is particularly acute in the case of algae as well. Nonetheless, the potential use of this method is very exciting although no studies, to date, have been attempted in plants or algae [37, 38].

5.2 Restricted proteolysis as an approach to target identification

Restricted proteolysis is a method commonly used to study conformational changes in protein structure as these structural shifts will change the exposure of the target site on the protein to the protease. Additionally, complex formation between a drug or other compound and a protein renders the complex more resistant to proteolytic cleavage. Exploiting this principle, Lomenick et al. developed a method called Drug Affinity Responsive Target Stability (DARTS) for target identification (Figure 6(a) [140, 147]. Briefly, a cell extract is treated with or without compound and then a protease such as thermolysin, subtilisin or pronase is added for a short duration to achieve limited proteolysis. The enriched target protein is subsequently detected using western blotting or mass spectrometry. The DARTS method was defined using known drug targets

including Rapamycin bound to mTOR, alpha-ketoglutarate to ATP synthase subunit, Didemnin B to EF1-alpha; and FK506 to FKB12 [140, 148]. In another study, potential targets of grape seed extract components protective in colorectal cancer were identified using DARTS coupled with a mass spectrometry-based spectral counting method [142].

Although the DARTS method is seemingly easy to implement, significant assay optimization is required including prior determination of the amount of protease enzyme to use and duration of the protease treatment. This can be simplified if the primary goal is target validation but is quite daunting for target discovery.

Recently another quantitative MS-based technique called Stability of Proteins from Rates Of Oxidation (SPROX) has been described. Similar to DARTS, this technique relies on the increased stability of compound-target complexes but focuses on changes in stability under oxidative conditions rather than resistance to proteases or thermal stability [141] (Figure 6(b)). SPROX assesses the thermodynamic properties of protein and protein-drug complexes upon hydrogen peroxide-mediated oxidation of methionine residues as a function of the chemical denaturant (e.g. guanidine hydrochloride or urea) concentration. SPROX is compatible with ion-exchange or other fractionation techniques used in MS-based proteomics analysis. Employing this technique, MS intensity of the peptides is used to generate thermodynamic information about the folding/refolding of the protein of interest induced by a denaturant such as urea/guanidine HCl. The oxidation reaction that occurs in SPROX is irreversible in nature. Isobaric tags such as tandem mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ) are useful in analyzing pooled samples treated under different denaturant concentrations. The reporter ion intensities from the peptides (oxidized/non-oxidized) are used to generate the chemical denaturation curves in SPROX. Proteins showing stabilization in the presence of compounds can be deemed to be direct binding partners of the compound/drug. An important disadvantage of this technique, as with other mass spectrometry technique, is the limited dynamic range imposed by the highly abundant proteins within the complex mixture and low affinity binding of the compound to non-target proteins. Compounds that are not stable in H_2O_2 may pose problems as they have limited solubility in aqueous buffer systems. Moreover, certain proteins such as those anchored within lipid bilayers may be resistant to the denaturant used for this experiment.

6 Case studies of chemical genetic approaches to improve lipid yield in plants and algae

The field of chemical screening to induce desired phenotypes is still in its infancy. Primarily developed and refined for mammalian cells and cell-free systems, there are relatively few successful applications in plant or algal systems. Reviewed below are four studies highlighting the potential applications of chemical genetics in plants and algae.

6.1 Case study 1: Strigolactones

Strigolactones are signaling molecules synthesized by plants. They have variety of functions such as control of plant development and, as a part of root exudates, they promote symbiotic interaction between the plant and the microbes in the soil. Moreover, parasitic weeds of genera *Striga* and *Orobanche* may reduce agricultural yield in the field. Generally, the seeds of the weed can remain dormant in the soil for years but as soon as they sense strigolactones in the vicinity, they are induced to germinate [149]. This makes strigolactones biosynthesis a major target to discourage parasitic plant growth. Several studies have attempted to use synthetic strigolactone analogs to induce suicide germination of the parasitic seeds but this had limited success owing to the high cost of synthesis and short half-life in soil. Using a chemical genetics screen in *Arabidopsis thaliana* screening 10,000 compounds resulted in identification of 5 structurally similar chemicals that inhibit cotyledon expansion and greening [150]. These structurally related compounds were later named cotylimides (CTLs) and it was determined that they function to increase the endogenous strigolactones levels. Further, using a genetic suppressor screen in *A. thaliana* against the CTL compounds, genes were identified that positively regulate strigolactone levels. The identification of the CTL compounds has facilitated screening for mutations that affects the strigolactones synthesis and signaling. Moreover, chemical screening has also been used to identify strigolactones antagonists. *In silico* screening of 4.7 million compounds (Library from Namiki Shoji Co. Ltd) was performed and 384 compounds were identified in the primary screen. Of these, 61 commercially available compounds were retested and 2-methoxy-1-naphthaldehyde (2-MN) was finally selected as a major inhibitor of strigolactones-dependent biochemical reactions in rice, *Arabidopsis* and *Striga* [151]. Thus, the identification of CLTs and subsequently 2-MN demonstrates the power and utility of chemical genetics in crop sciences.

6.2 Case 2: Identification of inhibitors of UDP-glucose and UDP-sugar pyrophosphorylase

UDP-sugars are substrates for several glycosyltransferase reactions and serve as a precursor for the biosynthesis of glycosylated compounds including starch. Both the UGP-glucose pyrophosphorylase (UGPase) and UDP-sugar pyrophosphorylase (USPase) have specificity for sugar-1-phosphates as substrates. Transgenic plants in which the expression of one or both UGPase genes was reduced using molecular genetic approaches, presented with male sterility or decreased number of seed [152, 153]. The products of USPase activity are required in a variety of glycosylation reactions that lead to the production of many metabolites and structural components in plants [154]. Deletion of the gene encoding this enzyme in *Arabidopsis* results in male sterility. Reverse chemical genetics was used to identify specific inhibitors of both UGPase and USPase. In this case, a chemical library of 17,500 compounds from the Chemistry Department of Umea

University, Sweden, was used to screen for inhibitors of both UGPase and USPase and 13 compounds that inhibits both UGPase and USPase were selected [155]. The most effective compound in inhibiting both UGPase and USPase was 6, 4H-1,3-benzoxazine-4-one. This compound was ineffective in inhibiting invertase, hexokinase, and glucose-6-phosphate dehydrogenase demonstrating specificity of action.

Chemical inhibition of UGPase in the diatom *Phaeodactylum tricornutum* demonstrated a role for this enzyme in carbon allocation. In this organism, the main storage polysaccharide is chrysolaminarin, which plays a role similar to that of starch in algae. Inhibition of UGPase led to a 24% increase in lipid content most likely due to channeling of carbon to lipid rather than starch [79]. In another study, a hyper-lipid producing strain was generated in *P. tricornutum* by deletion of UGPase using the transcription activator-like effector nuclease (TALEN) approach, which resulted in a significant increase in TAG [156]. Similar results were obtained in a starchless mutant of *Chlamydomonas* due to disruption of ADP-glucose pyrophosphorylase [24]. Thus, the use of UGPase and USPase inhibitors in algae are potential tools for improving lipid production in green algae and diatoms for biofuel production purposes.

6.3 Case study 3: Screening for modulation of growth, motility and photosynthesis in *C. reinhardtii*

A unique HTS of 5,445 compounds was performed using the microalgae *Chlamydomonas reinhardtii* to identify compounds that maintain growth fitness, motility and/or photosynthetic capacity [92]. Of these, approximately 44% (2,397) of the compounds altered algal growth after short term acute exposure (4-8 hours). Compounds that passed the first screen were then further screened for effects on phototaxis and photosynthesis. This screening identified 144 compounds as motility modulators and 350 as photosynthetic inhibitors; 18 were common to both assays. Using chemical fingerprint similarity analysis, 4 clusters were identified based on unique patterns of the activity. The cluster I compounds predominantly inhibit growth while cluster II compounds reduce motility/phototaxis. Cluster III compounds are photosynthesis inhibitors. Compounds in Cluster IV inhibit growth and photosynthesis, as well as modulating motility. The results were further analyzed to generate a Naïve Bayes model that predicts the bioactivity in *Chlamydomonas* based on the chemical fingerprints generated from the screening data to predict fitness inhibitors *in silico*. This is valuable to predict, for example, compounds that might induce lipid accumulation due to severe stress. The entire screening data set is available for bulk download from http://chlamychem.utoronto.ca/.

6.4 Case study 4: Screening of small molecules for improving triacylglycerol production during growth in algae

The three studies discussed below are similar and presented together as each is directed towards identification of small synthetic molecules useful in improving TAG production in algae and diatoms. In the first study, Franz et al., selected a series of chemical triggers that increase triacylglycerol production in marine algae [37]. The compounds tested were specifically selected to target a specific class of enzymes such as various kinases, fatty acid synthase (FAS), and lipases, as well as defence and oxidative signalling proteins. A list of some of the compounds is presented in Table 4 and Figure 7. Using a 96-well microplate-based assay, growth was measured using absorbance, chlorophyll contents were determined using fluorescence, and lipid production was assessed using lipophilic dye Nile Red. A total of 52 bioactive molecules were screened against 4 marine microalgal strains (*Nannochloropsis salina*, *N. oculata*, *Nannochloropsis* sp. and *Phaeodactylum tricornutum*) at a final concentration of 20 µM. Sodium bicarbonate was provided in the growth media as a supplemental source of carbon. In the first phase of the assay, > 20% increase in the lipid production was considered a hit. Selected compounds including cAMP, forskolin and quinacrine were active in all 4 strains; some compounds including (-)-epigallocatechin gallate (EGCG), cycloheximide and PTP inhibitor showed varied response based on the different strains. To further assess the effects of the chemical triggers at larger scale, the compounds were added to 500-mL batch cultures. Among the compounds that stimulated growth were forskolin, cAMP, quinacrine, orlistat and ECGC. Most of the compounds were effective when they were added at the start of the experiment at low cell density. Increase in lipid productivity was confirmed using additional orthogonal techniques such as 1H NMR, MALDI-TOF and GC-MS analysis [37].

In another study, a phenotypic screen was performed in the diatom *Phaeodactylum tricornutum* using a focused small molecule library called the Prestwick Chemical Library (total 1200 compounds) [157]. This library contains compounds annotated with pharmacological information that includes prior knowledge concerning the primary metabolic targets. The primary screen identified 160 compounds which were reevaluated and ranked to obtain an initial list of 40 compounds, which were further tested in biological triplicate to identify the final list of 34 hit molecules. The compounds selected were known to affect cell division and signaling, membrane transport or sterol metabolism. The targets of sterol metabolism included components of the sterol biosynthetic pathway including hydroxymethylglutaryl-CoA reductase, sterol 14-alpha demethylase, farnesyl pyrophosphate synthase, oxydosequalene cyclase (OSC), and cytochrome P450 oxidase/hydroxylase (Table 5).

The third and most comprehensive large-scale screening effort for identification of activators of lipid storage in algae was completed by Wase et al [38]. In this study, a large library of compounds (ChemBridge Corp; 43,783 compounds) were tested for growth and lipid accumulation using the

freshwater model green algae *C. reinhardtii*. Briefly, using a fixed concentration of 10 µM for the primary screening, 5 X 10^5 cells per well were exposed to the compounds and allowed to grow for 72 h in 384-well microplates then the lipophilic dye Nile Red was added to assess the lipid productivity and optical density at 600 nm was measured for assessment of growth. For quality control purposes, a Z-factor was measured to assess the dynamic range of the assay between vehicle control treated cells (growth without lipid production) and N-starved cells (no growth but with lipid production). Approximately, 0.079% of the compounds had a severe inhibitory effect on growth and about 3% of the compounds (1294) had a moderate inhibitory effect. The primary screen selected 367 compounds that induce lipid production at least 2-fold over untreated (DMSO treated) controls (Figure 3 Panel B, C, D). The screening hits were confirmed by retesting over a range of concentrations from 0.25 to 30 µM and activity of 243 compounds was confirmed. Based on the 243 actives, a network similarity model was constructed using chemical fingerprints and a Tanimoto cutoff of 0.7 for structure-based clustering. The hit compounds were classified into 5 structural groups based on the following common features: Group 1contain a piperidine moiety; Group 2 a benzylpiperazine moiety; Group 3 a nitrobenzene moiety; Group 4: phenylpiperazine moiety and Group 5 an adamantane moiety. (See Figure 8 and Table 6)

For further characterization, 15 high ranking lipid inducers were extensively examined for impact on cell growth, lipid productivity, starch accumulation, total proteins and photosynthetic pigments (chlorophyll a, b and total carotenoids). Among these, subset of the selected compounds had a limited inhibitory effect on growth after 48 hours post exposure. None of the compounds significantly reduced total cellular protein levels. This is in contrast to what is generally observed with classic lipid induction methods using stress conditions such as N starvation, which may result in protein loss as high as 60% [32]. Also, of high significance was the fact that 7 of 15 increased starch content, while one compound, WD30030, significantly reduced starch levels as compared to the untreated control. Only 2 compounds, WD10784 and WD10615, reduced chlorophyll a, chlorophyll b and total carotenoids levels significantly by about ~ 20 to 30%.

To further understand the impact of the selected lead compounds on lipid accumulation, a subset of 5 (chosen to represent the various structural classes) were assessed for fatty acid and complex lipid levels and composition by GM-MS and LCMS, respectively. Fatty acid analysis revealed that most of the compounds induced significant increases in the levels of C16:0, C18:1(Δ9), C18:2(Δ9,12) C18:3 (Δ5,9,12). Targeted complex lipid analysis (using LC-MRM/MS) revealed that total TAG content was increased more than 2.5-fold in cells treated with any of the 5 compounds. Importantly, there was no significant decline in galactolipids levels in cells with 4 of the 5 compound treatments. Since galactolipids are a major component of thylakoid membranes, this indicates the origin of the stored fatty acids and TAGs is not from plastid degradation or turnover.

This conclusion is supported by the maintenance of photosynthetic pigments and is in direct contrast to what occurs in N-deprivation induced lipid accumulation [158].
Using the same 5 compounds, cellular intracellular polar metabolite levels were profiled using GC-MS. Multivariate analysis, demonstrated overlapping and distinct impacts of each compound on central metabolism. There was a significant increase in accumulation of glucose-6-phosphate and fructose-6-phosphate compared with vehicle treated controls. These metabolites are proposed to drive carbon flux toward lipid synthesis. It was also shown that most amino acid levels were either maintained or significantly increased in abundance. This is in direct contrast to what occurs in N-deprivation where amino acid levels are reduced [30]. To test the general utility of these lipid inducing compounds, these compounds were tested in 3 additional freshwater algae, *Chlorella vulgaris* UTEX395, *C. sorokiniana* UTEX1230, and *Tetrachlorella alternans* UTEX2453, which are more relevant for commercial biofuel production. Similarly, the compounds induced significant increases in lipid production in a dose-dependent manner. (Please see Table 6 and Figure 8 and reference [38]).

This is indeed an exciting new area of research for algal biologists whereby chemical genetics can be successfully applied in both targeted [37] and unbiased screening [38, 157] of small compounds to select for those that induce TAG production in microalgae without severe stress that limits growth and biomass. Further, the results of these 3 studies in microalgae from diverse niche habitats (marine and freshwater) demonstrate the power and utility of chemical genetics to select compounds that induce a useful phenotype such high lipid production. Additionally, metabolomics analysis suggests other products may be enriched dependent upon the channeling of metabolite substrates into various pathways, thus opening the door to select compounds that help to produce a variety of high value products in a low cost sustainable manner.

7 Limitations / Bottlenecks of chemical screening in algae

Chemical-genetics techniques, while extremely powerful in perturbing cellular targets and inducing a phenotype of interest, has been employed in a very limited number of cases as reviewed above. A challenge of this technology is that, for non-targeted in vivo approaches, identification and validation of the ultimate target causing the desired phenotype is difficult and requires sophisticated biochemical, biophysical and/or immunochemical methodologies. Immunological approaches are constrained by the few antibodies available that interact specifically with algal antigens. Thus, identification will depend on novel approaches including synthesizing compound derivatives that generate affinity ligands detectable by mass spectrophotometry and other biophysical methods.

The second bottleneck for routine deployment of chemical genetics in algae is the prohibitively high cost of chemical library screening. To aid in the initial screening efforts, as reviewed above, investigators may employ the services of screening centers that are open to academic researchers. Moreover, commercial companies offer pre-defined libraries to screen hundreds rather than thousands of compounds thereby minimizing the initial cost of screening and facilitating screening method development. To popularize chemical genetics as a standard technology, efforts are needed to provide easy access to chemical libraries and expertise to exploit them. Websites such as PubChem and ChEMBL are filling part of this gap and researchers can mine these websites for information.

The third bottleneck is limitations to cultivation of photosynthetic organisms in microtiter plates as required to screen large numbers of unique compounds. One concern is poor aeration. This can be improved by using microplate membranes thereby minimizing contamination while allowing gas exchange. Regarding light delivery, LED lights should be used for microplates since they do not produce heat in the form of infrared (IR) radiation compared with fluorescent light sources. This will also minimize both the evaporation of media from the microplate well and temperature fluctuations.

Finally, one of the major limitation to algal chemical screening and studying the induced phenotypes is need for more powerful instrumentation and computational tools. These are required to permit full automation of large scale algae-based screens, including phenotypic selection and microscopy-based screening. Currently commercially available high content confocal microscopes are designed mainly for mammalian cell drug screens and must be adapted to algal/plant systems. It is also noted that chemical screening in academic laboratories is a complementary avenue to industrial pursuits. Academic researchers tend to operate outside of the commercial development paradigm and are more focused on discerning mechanistic understandings of the biological phenomenon as required to provide information on potential targets. While industry possesses the resources and expertise to develop a biological product, academic researchers have greater exploratory freedom as they are less encumbered by the rapid pace of industrial product development. These complementary goals necessitate collaborative efforts which will ultimately aid in translating initial findings into tangible products.

8 Conclusions and future opportunities in algal chemical genetics

As Thomas Jefferson wrote, “The greatest service that can be rendered to any country is to add a useful plant to its culture”, citing grains and oils as examples [159]. Recent advances regarding the biochemistry and regulation of algal oil production using directed and chemical genetics as reviewed here lend a new vibrancy to fulfill the promise of renewable, low cost energy sources to

reduce the need for fossil fuels and other petroleum based products. Beyond triglycerides, these approaches are opening the door to innovative approaches to employing algae as producers of many biomolecules of commercial and societal value. This information database has increased exponentially as genomics technologies began to dominate the field of algal research and provide future opportunities to study these targets using orthogonal approaches such as chemical genetics. Chemical biology is a powerful complementary tool to select compounds that induce in cells or purified biochemical targets phenotypes and activities of interest. This approach can be very effective in overcoming redundancy and lethality associated with certain mutations, which is a hallmark of traditional genetics. There are limitations, however, that must be overcome including: careful experimental design to ensure specificity and selectivity of high throughput screens; choice of compound libraries that provide a wide range of chemical core structures suitable to penetrate cell walls; and the expense of library screening. Financial constraints in screening large chemical libraries may prevent wide use in most academic laboratories and commercial entities may not have the capital to invest in a large number of chemical screens. Partnering between government funding agencies and academic and commercial organizations are highly desired for this purpose. A parallel approach, using small libraries against known targets (e.g. critical enzymes from lipid metabolism or central carbon metabolism) is also useful and may be conducted at low cost. It must be emphasized, that the results so generated would help to define more extensive and mature hypotheses necessary to understand and exploit lipid metabolism of algal bio-manufacturies.

A major limitation of algal chemical genetics is understanding the chemically induced phenotype in mechanistic detail. This will require a more complete understanding of algal metabolism and genetics, novel cell-based assays, more powerful instrumentation, computational algorithms. Acquisition of this information is made further made possible by NextGen sequencing and other "omics" technologies. Additionally, the new techniques such as DARTS, CETSA and SPROX designed to address unbiased identification of the target that is responsible for a particular phenotype (e.g. increased TAG production) are feasible, although not yet employed in algae. Additionally, much information is being generated by employing large-scale quantitative proteomics (label-free or using isobaric peptide labelling techniques (iTRAQ/TMT) or data independent techniques such as SWATH-MS and metabolomics to understand complex pathway shifts associated with small compound treatments even when the primary target is unidentified [38]. Thus, the combination of chemical genetics and new "Omics" techniques, the mechanism and specific targets of chemically induced phenotypes can now be probed at the intracellular levels.

Another major limitation in applying these approaches to the microalgae and other microorganisms is the limited understanding of the organisms' physiology, biochemistry and genetics. Although collections of genetic variants are available for mammalian, mouse, *Drosophila* and *Arabidopsis*

useful to design cell-based assay systems, these are not available for the microalgae or other microorganisms of commercial interest (e.g. cyanobacteria, diatoms). However, this can be improved by the generation of a collection of algal bioactive molecules related to general lipid metabolism and molecules that target the central carbon metabolism. Moreover, public repositories of chemical screening data for photosynthetic organisms is limited at this time and it is suggested that deposition of screening and chemical structure data to such repositories be required for publication of these results.

In summary, chemical genetics has gained considerable momentum as a tool to probe algal biology and to fulfill the promise of these organisms as low cost, high value, sustainable feedstock for biofuels and other high value products. These methods can be directed to identify molecules that induce or potentiate a natural biological process, which allows us to address basic mechanistic questions about the organism and its response to its environment, as well as offering opportunities to employ algae in a variety of commercial and environmental purposes. These include, but are not limited to production of: oils for fuel and nutrition; anti-oxidants; pigments, vitamins and medicinal products.

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Box 1: Quality control (QC) and hit identification methods

Percent inhibition/activation cut-off: For identification of "hits" using this method, the screening data is first normalized and then an arbitrary cut-off value is assigned that is relative to the assay signal window. This method does not have any statistical basis and is primarily used in small molecule screens with strong controls.

Average +/- 3 SD: In this method, cut-off is generally set to a value that is 3 times standard deviation above or below the sample average [160]. Because of the use of average and standard deviation, this method is sensitive to outliers.

Median +/- k MAD: to blunt the effect of outliers, it was proposed that a cut-off of k MAD should be used to identify 'hits'. A recent study has shown that MAD-based hit selection strategy has lower false discovery rate as compared to the mean +/- SD method [161].

Percent of control (POC): is a normalization method where compound treatment outcome is normalized relative to the control. For example, the raw measurement for each treated sample is divided by the average of the control values given by following equation.

$$\text{Percent of control (POC)} = \frac{x_i}{\bar{c}} \times 100$$

where, x_i is the raw intensity (optical readout) of the i^{th} compound and $\bar{c}$ is the mean intensity of the control.

Normalized fold change (NFC): NFC is a control based method where ratio of compound treatment outcome and the mean of control are divided by the difference in the control and the compound measurements.

$$\text{Normalized Fold Change (NFC)} = \frac{x_i \div \bar{c}}{\bar{c} - x_i}$$

But if two measurements were recorded such as fluorescence intensity for lipid accumulation (Nile Red fluorescence) and optical density of the cells, then the NFC can be calculated using following formula (as used in a recent study [38]).

$$\text{Normalized Fold Change (NFC)} = \frac{x_{NR} \div \overline{C_{NR}}}{x_{OD} \div \overline{C_{OD}}}$$

where, x_{NR} is the Nile Red fluorescence of the compound X and C_{NR} is the average Nile Red fluorescence of negative control, X_{OD} is the optical density of compound X and C_{OD} is the average optical density of negative control.

Z-factor: Z-factor is a dimensionless measurement where both positive and negative control are used. Generally, 4 parameters are used for calculating Z-factor: the mean (μ) and standard deviations (∂) of both positive and negative controls are used [162].

$$Z'\text{-factor} = 1 - \frac{(3\partial pc + 3\partial nc)}{|\mu pc - \mu nc|}$$

Where, ∂_{pc} = standard deviation of positive control; ∂_{nc} = standard deviation of negative control; μ_{pc} = mean of positive control and μ_{nc} = mean of negative control. The Z'-factor can ideally never exceed 1. High Z'-factor > 0.5 defines a robust assay, while between 0 and 0.5 defines a marginal assay. If the Z'-factor is less than 0, there is too much overlap between the positive and negative controls and the assay is invalid.

Strictly Standard Mean Difference (SSMD): This method has been widely used for RNAi screening data since it has a statistical basis and has a better control on false negative and false positive rates. This method captures effect size. SSMD has the ability to handle controls with different effects. Although SSMD is primarily used for RNAi screen but can be used for small molecule screen as well [163]

$$SSMD = \frac{\text{mean}(C_{pos}) + \text{mean}(C_{neg})}{\sqrt{\text{std}(C_{pos})2 + \text{std}(C_{neg})2}}$$

BScore: Normally in a large-scale screening experiment, both positive and negative invariably shows variability since average values and standard deviation are generally influenced by statistical outliers, which in current situation are potential hits. To circumvent this problem, a more robust analysis method such as B-score can be applied [164]. The B-score is a method similar to Z'-score but uses dispersion index and this method is more resistant to outliers and error measurement distributions. The residual (r_{mnx}) of a compound at row *m* and column *n* on the plate *x* can be obtained by following equation as provided by [100]:

$$r_{mnx} = Y_{mnx} - \hat{Y}_{mnx} = Y_{mnx} - (\widehat{\mu_x} + \widehat{R_{mx}} + \widehat{C_{nx}})$$

The residual (r_{mnx}) is the difference between the observed result (Y_{mnx}) and the fitted value ($\hat{Y}_{mnx}$) defined as estimated mean value of the plate ($\hat{\mu}_x$)+ estimated systematic measurement offset for m^{th} row on plate *x* ($\widehat{R_{mx}}$) + estimated systematic measurement offset for n^{th} column on plate *x* ($\widehat{C}_{nx}$)). For each plate x, the adjusted median absolute deviation (MAD_x) is obtained from the r_{mnx} (MAD_x). Thus, the B-score can be calculated as:

$$Bscore = \frac{r_{mnx}}{MAD_x}$$

For calculation of the B-score, first Tukey's two-way median polish is calculated to account for both column and row effect within each plate. The resultant residual (r_{mnx}) is then divided by the median absolute deviation to standardize each plate and account for the inter- and intra-plate variation. Thus, the B-score is advantageous as it is non-parametric, it minimizes measurement bias due to position effect and is resistant to outliers.

Figure legends:

Figure 1: Schematic representation of lipid biosynthesis in microalgae. AGPase ADP-glucose pyrophosphorylase; AGPAT 1-acylglycerol-3-phoshphate acetyltransferase; ACL ATP citrate lyase; CS citrate synthase; DGAT Diacylglycerol acyltransferase ;GPD2 glycerol-3-phoshphate dehydrogenase; GPAT acyl-CoA:glycerol-3-phosphate acyltransferase; MCAT malonyl coenzyme A:acyl carrier protein transacylase; PAP phosphatidic acid phosphatase; PDAT phospholipid:diacylglycerol acyltransferase; PDH Pyruvate dehydrogenase; PK pyruvate kinase

Figure 2: Comparison of classical and chemical genetic screening in algae. A) Forward genetic screening generally involves performing chemical mutagenesis of a population of cells and then screening for a phenotype of interest. B) Forward chemical genetics involves treating cells with a single chemical from a synthetic compound library and performing phenotypic selection. After identification of a "hit" compound, the target is identified using specialized strategies (see text section 6). C) Reverse genetics involves directed mutagenesis of a specific gene followed by assessment of a desired activity or phenotype. D) Reverse chemical genetics involves the use of small molecules targeting a single protein**.**

Figure 3: Chemical screening plate layout and data analysis A) Normally commercial chemical libraries are provided as 96- or 384-well plate format. In a 96-well format, 80 compounds are plated in the middle and first and last columns are kept empty for plating the controls. Eight positive and 8 negative controls are plated in a typical 96-well plate. For a typical assay, during the primary screen, first the compounds are added to desired final concentration in wells (indicated by green circles), then a fixed number of cells are added to each well. For the negative control (blue circles) instead of the compound, the same volume of solvent (for example DMSO) is added; in the first column, a positive controls is placed (red circles). Plates are incubated or other manipulations done and then activity is measured for every well by automated plate reading. **B)** Results of Z-factor calculation to assess the assay quality. Generally Z-factor > 0.5 indicates good quality of assay. **C)** Growth data of *Chlamydomonas* screening of 43,000 compounds for lipid inducers. **D)** Representative screening output after normalization and ratio calculation for lipid inducers. Lipid accumulation measured as relative fluorescence after Nile Red (NR) staining of cells treated with compound relative to cells treated with vehicle (DMSO). Using a cut-off of 2.5-fold, 243 compounds were identified as "hits" or lipid inducers (highlighted in yellow). Data for panel B, C & D is from Wase et al. (2017) [38].

Figure 4: Schematic map of central carbon and lipid metabolism showing possible targets for inhibition or activation. Metabolic steps are represented by arrows. Genes encoding the enzymes are labeled in red. Chemical inhibitors of enzymes are shown in orange boxes while chemical activators are shown in green boxes. Abbrevations: AGPase ADP-glucose pyrophosphorylase; AGPAT 1-acylglycerol-3-phoshphate acetyltransferase; DGAT Diacylglycerol acyltransferase ;

ENO Enolase; FUM Fumarate; G3PDH glycerol-3-phosphate dehydrogenase; GK Hexokinase/glucokinase; GPAT acyl-CoA:glycerol-3-phosphate acyltransferase; IDH Isocitrate dehydrogenase; MCAT malonyl coenzyme A:acyl carrier protein transacylase; MDH Malate dehydrogenase; PAP phosphatidic acid phosphatase; PDAT phospholipid:diacylglycerol acyltransferase; PDH Pyruvate dehydrogenase; PEPC phosphoenoylpyruvate carboxylase; PGK Phosphoglycerate kinase; PK pyruvate kinase; SDH Succinyl dehydrogenase

Figure 5: **Schematic workflow of CETSA method**. Cells are grown and cell lysate was obtained. The cell lysate was treated with either drug or vehicle (DMSO), and aliquots are subjected to heating. After cooling, the soluble protein fraction is separated from precipitated proteins by centrifugation. The abundance of the native target proteins can be analyzed by either antibody-based western blotting method or by MS-based approaches. Figure adopted from [146] with permission from the publisher.

Figure 6. Comparison of two methods for target identification. (A) DARTS workflow. Cell lysates are treated with vehicle or compound, and then subjected to limited proteolysis. Target proteins are stabilized against proteolysis by bound compound, and thus enriched. The samples are then analyzed by either immunoblot (target validation) or mass spectrometry (target identification). B) Schematic representation of the SPROX experimental workflow. A complex protein mixture (e.g., a cell lysate) is subjected to two treatments, with and without drug. In each analysis, aliquots of the protein mixture are diluted into buffer containing increasing concentrations of a chemical denaturant guanidinium hydrochloride (GdmCl). The protein samples in each GdmCl-containing buffer is then allowed to react with a fixed amount (30%) of hydrogen peroxide for a limited time. The reaction time and concentration of hydrogen peroxide are tuned such that the thioether groups in the side chain of methionine residues are selectively oxidized. The protein oxidation reaction is stopped by adding catalase enzyme. The protein samples in each tube are then further reduced, alkylated, digested by trypsin, and labelled with iTRAQ 8-plex reagents (from isobaric label 113 to 121). Finally, the samples are pooled together. Samples can be optionally fractionated using Strong cation exchange or HILIC chromatography and submitted to quantitative proteomic analysis. The non-oxidized and oxidized methionine-containing peptides are quantified as a function of the SPROX buffer denaturant concentration. Proteins that are stabilized, destabilized or showing no effect can then be analysed. Adapted from reference [140] with permission from publisher.

Figure 7: Structures of compounds selected as inducers of lipid body accumulation in a phenotypic screen in 4 marine microalgal species. Structure information was obtained from Franz et al(2013) [37].

Figure 8: Structures of compounds selected by lipid accumulation in a large high throughput screening in the green algae *Chlamydomonas reinhardtii*. Structure information was obtained from Wase et al(2017) [38].

Table 1. Summary of studies employing directed molecular engineering methods to enhance lipid production

Genetic method	***Protein***	***Functional role***	***Engineered algae***	***Outcome***	***References***
TCA enzymes					
Knockdown	Phosphoenolpyruvate carboxylase 1, PEPC1	Formation of oxaloacetate from phosphoenolpyruvate and regulates carbon flux	*Chlamydomonas reinhardtii*	28.7–48.6% increased TAG	[71]
RNA interference	Citrate synthase (CS)	Regulates C flux to citrate synthesis or TAG	*Chlamydomonas reinhardtii*	169.5% increase d TAG	[27]
Lipid Metabolism					
Overexpression	ACCase	Catalyzes malonyl-CoA formation	*Cyclotella cryptica*	No improvement in TAG levels	[70]
Overexpression	ACCase	Catalyzes malonyl-CoA formation	*Navicula saprophila*	No improvement in TAG levels	[69]
Overexpression	KAS 2	FA biosynthesis regulation	*Chlamydomonas reinhardtii*	Increase in C18 fatty acids (lipid quantitation not known)	[72]
Overexpression	myristic acid thioesterase (C14-TE)	Hydrolyzes acyl-ACP to release free FAS	*Phaeodactylum tricornutum*	C 14:0 increased 15%	[73]

Overexpression	lauric acid thioesterase (C12-TE)		*Phaeodactylum tricornutum*	C 12:0 increased 6.2%	[73]
Overexpression	Lauryl-ACP thioesterase and FatB1 thioesterase		*Chlamydomonas reinhardtii*	No change	[72]
Overexpression	stearoyl-ACP desaturase	Catalyzes the conversion of stearic acid (18:0) to oleic acid (18:1)	*Chlamydomonas reinhardtii*	Total FA increased by 28%	[66]
Overexpression	microsomal Δ12-desaturase	Catalyze conversion of oleic acid to linoleic acid	*Nannochloropsis oceanica*	arachidonic acid increased by 50%–75%	[67]
Knockdown	long-chain acyl-CoA synthetases (LACSs)		*Chlamydomonas reinhardtii*	45-55% increased total lipids	[165]
TAG biosynthesis enzymes					
Overexpression	DGAT2	Responsible for addition of acyl group at sn-3 position of DG	*Phaeodactylum tricornutum*	35% increased TAG and 76.2% increased EPA	[166]
Overexpression	DGAT2-1; DGAT2-5		*Chlamydomonas reinhardtii*	20% and 44% increase in the DGAT2–1 and DGAT2–5 overexpression lines, respectively	[59]
Overexpression	DGAT2-a,b,c		*Chlamydomonas reinhardtii*	No significant change	[60]
Overexpression	DGAT2		*Thalassiosira pseudonana*	1.52 to 1.95-fold increased TAG	[167]
Overexpression	DGAT4		*Chlamydomonas reinhardtii*	1.5 to 2.5-fold increased TAG	[168]
Overexpre	G3PDH +	TAG biosynthesis pathway enzymes	*Chlamyd*	Two-fold increased TAG	[74]

ssion	GPAT + LPAT + PAP + DGAT		*omonas minutissima*		
Knock down	PDAT1	acyl-CoA–independent enzyme PC -> TAG	*Chlamydomonas reinhardtii*	25% decrease in TAG	[58]
Overexpression	GPAT	acylation of glycerol 3-phosphate by glycerol-3-phosphate acyltransferase	*Chlamydomonas reinhardtii*	50% increase in the TAG	[26]
	TAG storage and lipid body formation				
RNA interference	MLDP (major lipid droplet protein)	Major Lipid droplet protein	*Chlamydomonas reinhardtii*	Increased LD size with no changes in TAG content	[169]
	PNPLA3	Membrane bound protein associated with LDs	*Phaeodactylum tricornutum*	70% increase TAG	[76]
amiRNA repression	Nitrogen regulatory protein PII protein	Negatively controls TAG accumulation in LDs during nitrogen starvation	*Chlamydomonas reinhardtii*	increased LDs and a 1.5-fold increased TAG	[170]
	Transcription factors				
Overexpression	bZIP transcription factor	bZIP TF is stress regulator and associated with lipid metabolism	*Nannochloropsis salina*	203% increased FAME productivity under high salt stress	[68]
Overexpression	DOF-type transcription factor	Involved in FA and glycerolipid biosynthesis regulation	*Chlamydomonas reinhardtii*	A 2-fold increase of total lipids	[85]
CRISPR-Cas9 + RNAi interference	Zn_2Cys_6 transcription regulator	Zn/cys activates the transcription of genes involved in galactose and melibiose metabolism	*Nannochloropsis gaditana*	Improve C partition to lipids and doubles lipid production in N replete condition.	[25]
Overexpression	AtWR1 transcription factor	transcription factor WR1 controls seed oil accumulation in Arabidopsis thaliana,	*Nannochloropsis salina*	Increased total lipids by 36.5 and 44.7%	[86]
Overexpre	Sepin	Sepin regulates the activity of GPAT	*Phaeoda*	Increased TAG by 57%	[35]

ssion	overexpressio n		*ctylum tricornut um*		
		Other targets			
Overexpre ssion	NAD(H) kinase	Catalyzes synthesis of NADPH	*Chlorell a pyrenoid osa*	110.4% increase in lipid levels	[48]
Repression	NRR1	Transcriptional regulation of lipid biosynthetic genes under N-deprivation	*Chlamyd omonas reinhard tii*	50% decrease in TAG during nitrogen-deprivation	[58]
RNA interferenc e	AMP deaminase	converts adenosine monophosphate (AMP) to inosine monophosphate (IMP)	*Chlamyd omonas reinhard tii*	25% higher lipid accumulation	[77]
Knockdow n	chrysolaminari n synthase	catalyzes the synthesis of glucan using UDP-glucose as a substrate	*Thalassi osira pseudon ana*	Increased TAG > 2-fold	[78]
Knockdow n	ADP glucose phosphorylase	Rate limiting enzyme of starch synthesis	*Chlamyd omonas reinhard tii*	Increased TAG by 7-fold	[24]
Overexpre ssion	Haematococcu s oil globule protein (HOGP)	Protein associated with LDs	*Phaeoda ctylum tricornut um*	25% increase in total FA	[171]
RNAi interferenc e	UDP-glucose pyrophosphor ylase (UGPase)	catalyzing the reversible production of UDP-Glc and pyrophosphate (PPi) from glucose-1-phosphate (Glc 1-P) and UTP	*Phaeoda ctylum tricornut um*	45% increase in TAG	[79]

Table 2: List of screening library collections.

Chemical library Collection	**Website**	**Type**

The NExT Screening Libraries	https://next.cancer.gov/discoveryResources/resources_ndl.htm	Academic
Stanford High Throughput Bioscience Center	http://htbc.stanford.edu/compounds.html	Academic
Charles River Compound Screening Libraries	https://www.criver.com/products-services/discovery-services/assay-development-and-screening/compound-screening-libraries?region=3601	Commercial
Prestwick Screening libraries	http://www.prestwickchemical.com/libraries-screening-libraries.html	Commercial
Boston University Chemical Library Consortium (CLC)	http://www.bu.edu/cmd/center-overview/biology-outreach/	Academic
ChemBridge Corp	http://www.chembridge.com/screening_libraries/	Commercial
Milner Therapeutics Institute (University of Cambridge)	https://www.milner.cam.ac.uk/consortium/	Academic/commercial
Otava Agrochemical libraries	http://www.otavachemicals.com/products/screening-compounds-for-agrochemical-discovery	Commercial
LATCA (University of California Riverside)	http://www.thecutlerlab.org/2008/05/latca.html	Academic
Maybridge Hitfinder collection	https://www.maybridge.com/	Commercial
Life Chemicals Screening libraries	http://www.lifechemicals.com	Commercial
Analyticon Discovery	https://ac-discovery.com/screening-libraries/	Commercial
Academic Drug discovery consortium	http://addconsortium.org/interior-partnerships-az.php	Academic
PCBIS Plateforme de Chimie Biologie Integrative de Strasbourg (Screening Strasbourg Platform)	http://www.pcbis.fr	Academic
Broad Institute Chemical Biology Program	https://www.broadinstitute.org/chembio-therapeutics	Academic

Table 3: Selected commercial and open-access software packages for statistical analysis of HTS screening data

Tool	***Features***	***Programming language***	***Reference***	***Type***

HTS screening software				
Mscreen	Web-based compound library management and QC	PHP, Oracle	[172]	Open source
NextRNAi	design and evaluation of genome-wide RNAi libraries	Perl	[173]	Open source
K-Screen	Web-based application for chemical library, primary and secondary screening data	R/PHP, SQL	[174]	Open source
HTS corrector	Statistical analysis of HT-screening data	C#	[175]	Open source
HTSanalyzeR	HTS screening data analysis, gene set enrichment and network analysis	R/Bioconductor	[176]	Open source
HTSvis	Web based shiny application for HT-screening data analysis	R/Bioconductor	[177]	Open source
cellHTS2	Analysis of cell based screening	R/Bioconductor	[178]	Open source
RNAither	Statistical analysis of HTS-RNAi screen	R/Bioconductor	[179]	Open source
HiTSeekR	Web platform for analysis of HT-screening data	R/Bioconductor	[180]	Open source
bioassayR	cross-analysis of small molecule bioactivity	R/Bioconductor	[181]	open source
Tobco Spotfire	Analysis of large volume screening data and compound structure analysis		--	Commercial
Chemoinformatics software				
Tobco spotfire	Commercial software for chemical compound visualization, dose-response assay, library management		--	Commercial
HTS navigator	Windows application for chemoinformatics data analysis.	--	[182]	Open source
DataWarrior	Java based free chemoinformatics program for data visualization and analysis	Java	[183]	Open source

Chem Mine	Web-based tool for chemoinformatics analysis	R/Bioconductor	[184]	Open source
chemViz	Cytoscape plugin for chemoinformatics data analysis	Cytoscape plugin		Open source
Vortex	Interactive data visualization and analysis solution for combined chemoinformatics and bioinformatics analysis	--		Commercial

Table 4: Assessment of lipid induced in marine algae by chemicals with known targets and mechanisms of action (data based on [37].)

Compound	**Target**	**% increase NR fluorescence**	**Algae strain**	**Reference**
	Lipase inhibitors			
RHC 80267	Diacylglycerol lipase inhibitor	> 50	various marine algae	
Orlistat(tetrahydrolipstatin)	Lipase inhibitor	> 50	various marine algae	
JZL184 hydrate	Monoacylglycerol lipase inhibitor	> 70%	various marine algae	
Halopemide	phospholipase inhibitor	> 100%	various marine algae	
PTFK (Palmityl trifluoromethyl ketone)	Phospholipase inhibitor	> 70%	various marine algae	
ET-18-OCH3	inhibitor of phosphatidylinositol-specific phospholipase	> 50%	various marine algae	
	Antioxidant compounds			
BHA (Butylated hydroxyaniaole)	Antioxidant	> 250%	various marine algae	[37]
Resveratrol	Antioxidant, anticancer, antifungal, inhibitor of COX-1	> 250%	various marine algae	
Propyl Gallate	Antioxidant, inhibitor of	> 250%	various	

	microsomal lipid peroxidation		marine algae	
(-)-Epigallocatechin gallate	Antioxidant, cannabinoid receptor agonist	> 200%	various marine algae	
Apigenin	Plant hormone, CYP2C9 inhibitor	> 80%	various marine algae	
	Lipoxygenase inhibitors			
Caffeic Acid	LOX	> 200%	*P. tricornautum*	
Gossypol	LOX	> 200%	*P. tricornautum*	
	Protein tyrosine kinase inhibitors			
BPDQ	PTK	> 100%	*N. salina*	
Genistein	PTK	> 50%	*N. salina*	
Butein	PTK	> 80% (H2O)	*P. tricornautum*	
	Protein tyrosine phosphate inhibitors			
PTP inhibitor II	PTP	> 80%	*N. salina*	
Cantharidin	PTP	> 150% (H2O)	*P. tricornautum*	

Table 5: Selected compounds that induced lipid accumulation in the diatom *Phaeodactylum tricornutum* CCMP 2561. Data based on [157].

CHEMBL ID	Chemical Name	Target	TAG accumulation	Reference
CHEMBL959	Xylometazoline	Adrenergic receptor agonist	High NR signal	[157]
CHEMBL9514	Mevastatin	HMG-CoA reductase	High NR signal	
CHEMBL691	Simvastatin	HMG-CoA reductase	High NR signal	
CHEMBL606	Allopurinol	inhibitor of xanthine oxidase	High NR signal	
CHEMBL599	Nocodazole	Microtubule depolymerizing agent	High NR signal	

CHEMBL55	Rimantadine	Proton pump inhibitor	High NR signal	
CHEMBL54440	Alverine	Serotonin receptor antagonist	High NR signal	
CHEMBL429	Ethynylestradiol	Steroid hydroxylase substrate	High NR signal	
CHEMBL403	Estrone	Steroid hydroxylase substrate	High NR signal	
CHEMBL39	Ketoconazole	sterol 14-alpha demethylase inhibitor	High NR signal	

Table 6: Selected compounds that showed more than 2.5-fold lipid induction in green algae *Chlamydomonas reinhardtii*. The primary screen was performed at a single dose of 10 µM to identify 367 compounds as hits. The lipid accumulating capacity was further confirmed using a dilution series (dose-response assay) at concentration ranging from 30 µM to 0.25 µM. Table showing fold change values of lipid accumulating capacity (relative to vehicle treated control) from the primary and confirmatory screen. Data based on [38] for compounds tested in *Chlamydomonas reinhardtii*. Please see Figure 8 for the compound structures.

	Primary screen			
Compounds	*10 µM*	*30 µM*	*15 µM*	*10 µM*
WDTHQ130	2.28	20.71	20.86	11.98
WD40844	12.44	19.77	21.53	13.97
WD40157	2.51	18.58	25.48	10.34
WD30999	2.90	13.04	16.13	8.91
WD30030	2.90	21.10	15.48	4.46
WD20542	2.75	19.74	22.79	11.58
WD20067	2.54	19.83	18.82	8.70
WD10872	2.97	18.56	21.56	10.80
WD10784	2.14	13.69	21.46	3.63
WD10738	4.67	21.78	20.48	10.18
WD10615	2.97	12.47	18.52	9.49
WD10599	2.44	9.85	10.08	9.28
WD10461	4.38	21.07	19.19	8.95
WD10264	3.58	8.29	16.37	6.76
WD10256	2.18	12.43	14.61	7.30

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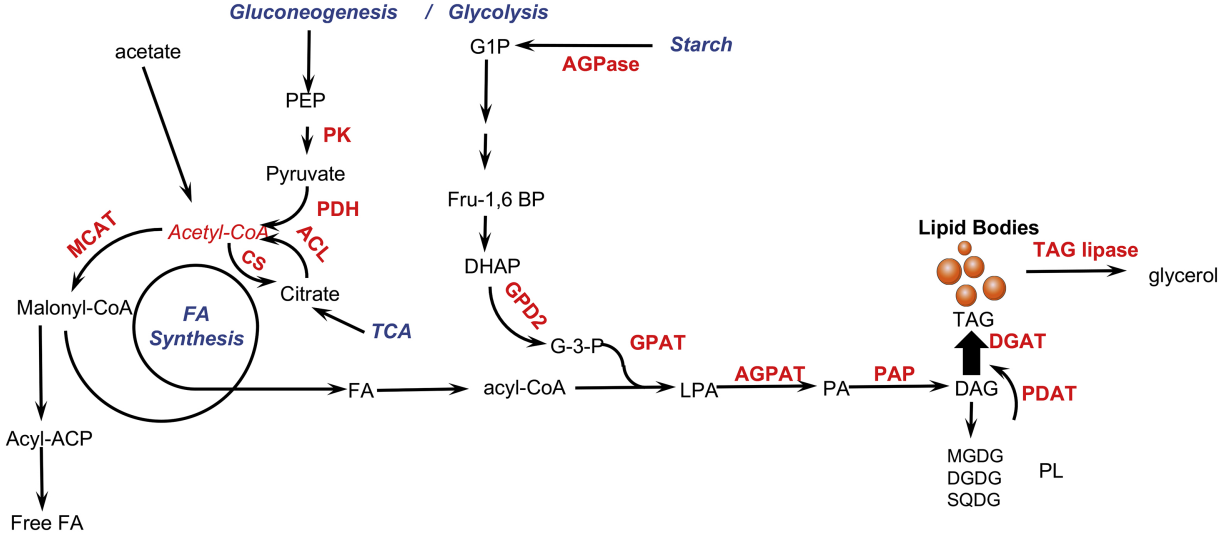

Gluconeogenesis / Glycolysis
acetate
PEP
PK
Pyruvate
PDH
Acetyl-CoA
MCAT
ACL
CS
Citrate
TCA
Malonyl-CoA
FA Synthesis
Acyl-ACP
Free FA
G1P
Starch
AGPase
Fru-1,6 BP
DHAP
GPD2
G-3-P
GPAT
FA
acyl-CoA
LPA
AGPAT
PA
PAP
DAG
PDAT
DGAT
TAG
Lipid Bodies
TAG lipase
glycerol
MGDG
DGDG
SQDG
PL

Figure 1

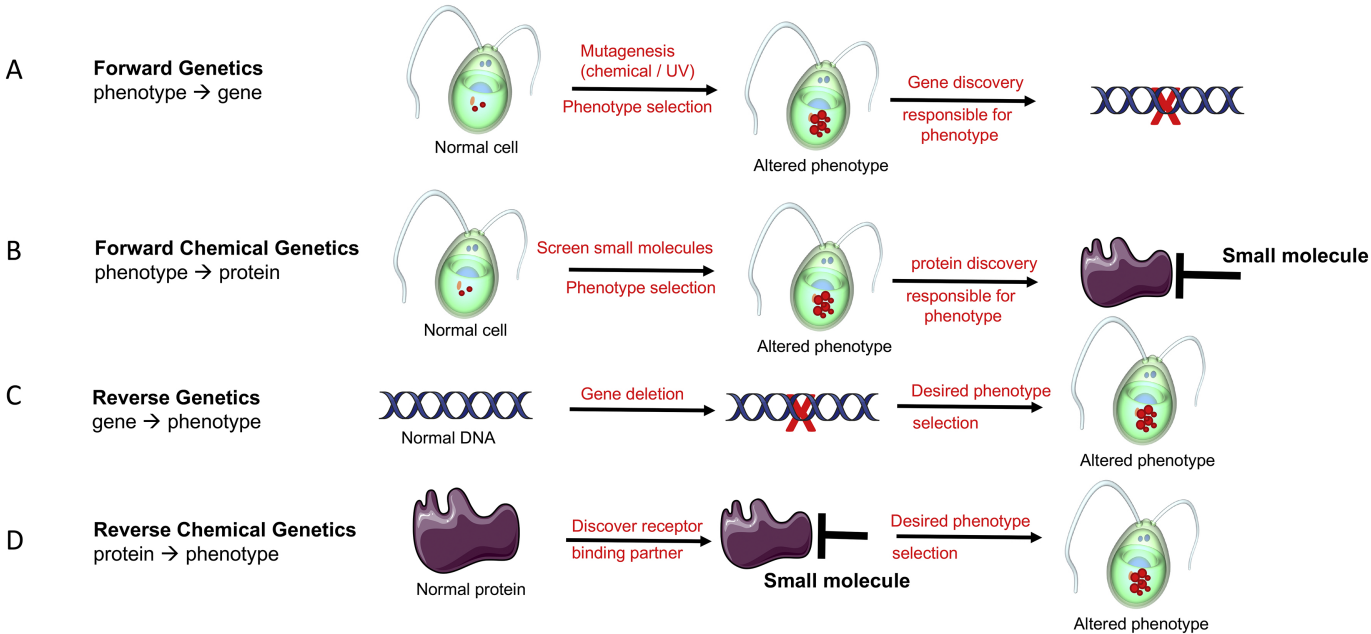

A
Forward Genetics
phenotype → gene
Normal cell
Mutagenesis
(chemical / UV)
Phenotype selection
Altered phenotype
Gene discovery
responsible for
phenotype
B
Forward Chemical Genetics
phenotype → protein
Normal cell
Screen small molecules
Phenotype selection
Altered phenotype
protein discovery
responsible for
phenotype
Small molecule
C
Reverse Genetics
gene → phenotype
Normal DNA
Gene deletion
Desired phenotype
selection
Altered phenotype
D
Reverse Chemical Genetics
protein → phenotype
Normal protein
Discover receptor
binding partner
Small molecule
Desired phenotype
selection
Altered phenotype

Figure 2

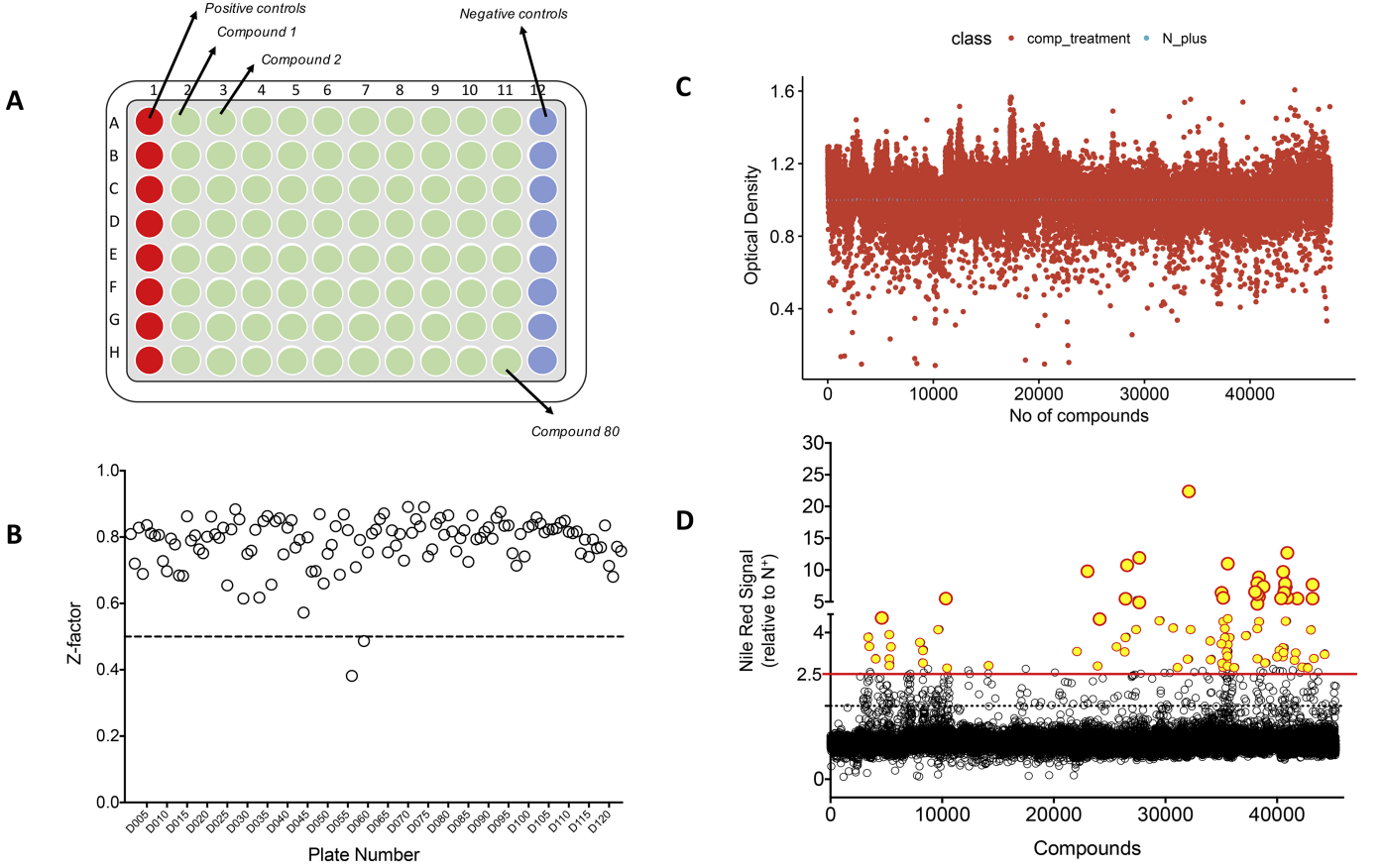

A
Positive controls
Compound 1
Compound 2
Negative controls
Compound 80
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
B
Z-factor
1.0 0.8 0.6 0.4 0.2 0.0
D005 D010 D015 D020 D025 D030 D035 D040 D045 D050 D055 D060 D065 D070 D075 D080 D085 D090 D095 D100 D105 D110 D115 D120
Plate Number
C
class comp_treatment N_plus
Optical Density
1.6 1.2 0.8 0.4
0 10000 20000 30000 40000
No of compounds
D
Nile Red Signal (relative to N+)
30 25 20 15 10 5 4 2.5 0
0 10000 20000 30000 40000
Compounds

Figure 3

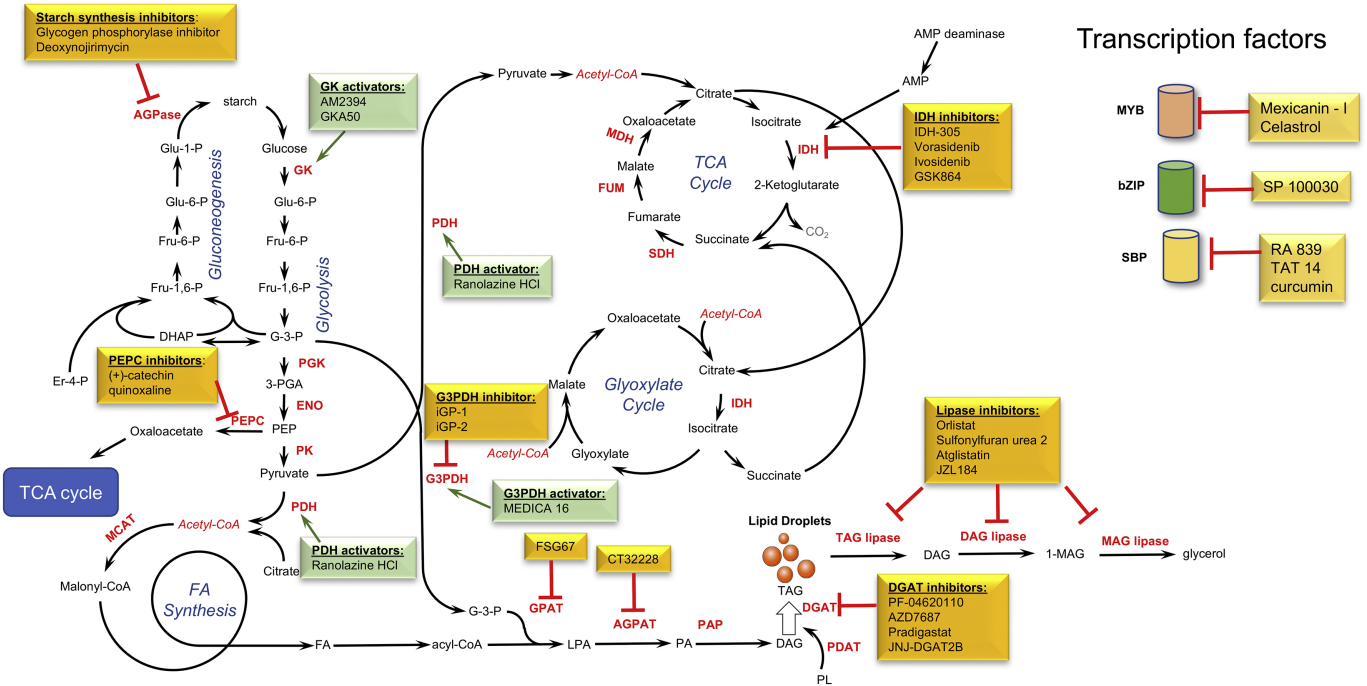

Starch synthesis inhibitors:
Glycogen phosphorylase inhibitor
Deoxynojirimycin
AGPase
starch
Glu-1-P
Glucose
GK activators:
AM2394
GKA50
GK
Glu-6-P
Fru-6-P
Fru-1,6-P
DHAP
G-3-P
Gluconeogenesis
Glycolysis
PEPC inhibitors:
(+)-catechin
quinoxaline
Er-4-P
PEPC
PGK
3-PGA
ENO
PEP
Oxaloacetate
PK
Pyruvate
TCA cycle
MCAT
Acetyl-CoA
PDH
PDH activators:
Ranolazine HCl
Citrate
Malonyl-CoA
FA Synthesis
FA
acyl-CoA
G-3-P
GPAT
LPA
AGPAT
PA
PAP
DAG
DGAT
PDAT
PL
Pyruvate
Acetyl-CoA
Citrate
Isocitrate
IDH
2-Ketoglutarate
CO2
Succinate
SDH
Fumarate
FUM
Malate
MDH
Oxaloacetate
TCA Cycle
PDH
PDH activator:
Ranolazine HCl
IDH inhibitors:
IDH-305
Vorasidenib
Ivosidenib
GSK864
AMP deaminase
AMP
G3PDH inhibitor:
iGP-1
iGP-2
G3PDH
G3PDH activator:
MEDICA 16
Oxaloacetate
Acetyl-CoA
Citrate
Isocitrate
IDH
Malate
Glyoxylate Cycle
Acetyl-CoA
Glyoxylate
Succinate
FSG67
CT32228
Lipase inhibitors:
Orlistat
Sulfonylfuran urea 2
Atglistatin
JZL184
Lipid Droplets
TAG
TAG lipase
DAG lipase
MAG lipase
DAG
1-MAG
glycerol
DGAT inhibitors:
PF-04620110
AZD7687
Pradigastat
JNJ-DGAT2B
Transcription factors
MYB
Mexicanin - I
Celastrol
bZIP
SP 100030
SBP
RA 839
TAT 14
curcumin

Figure 4

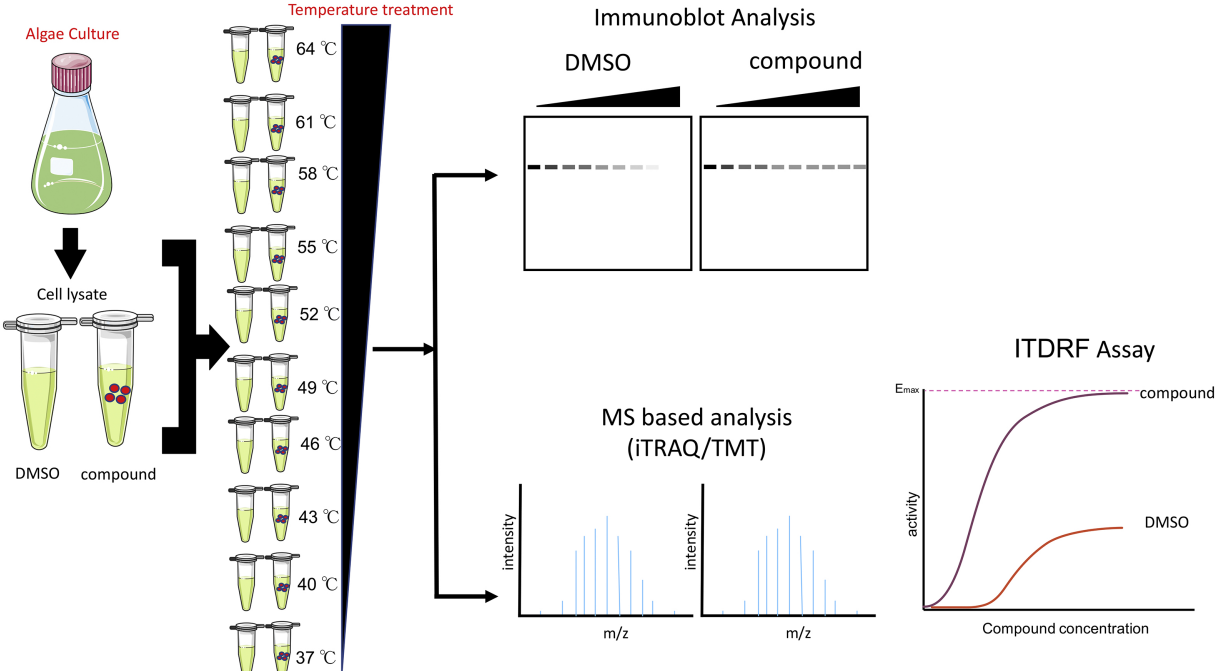

Algae Culture
Cell lysate
DMSO
compound
Temperature treatment
64 ℃
61 ℃
58 ℃
55 ℃
52 ℃
49 ℃
46 ℃
43 ℃
40 ℃
37 ℃
Immunoblot Analysis
DMSO
compound
MS based analysis
(iTRAQ/TMT)
intensity
m/z
intensity
m/z
ITDRF Assay
Emax
compound
DMSO
activity
Compound concentration

Figure 5

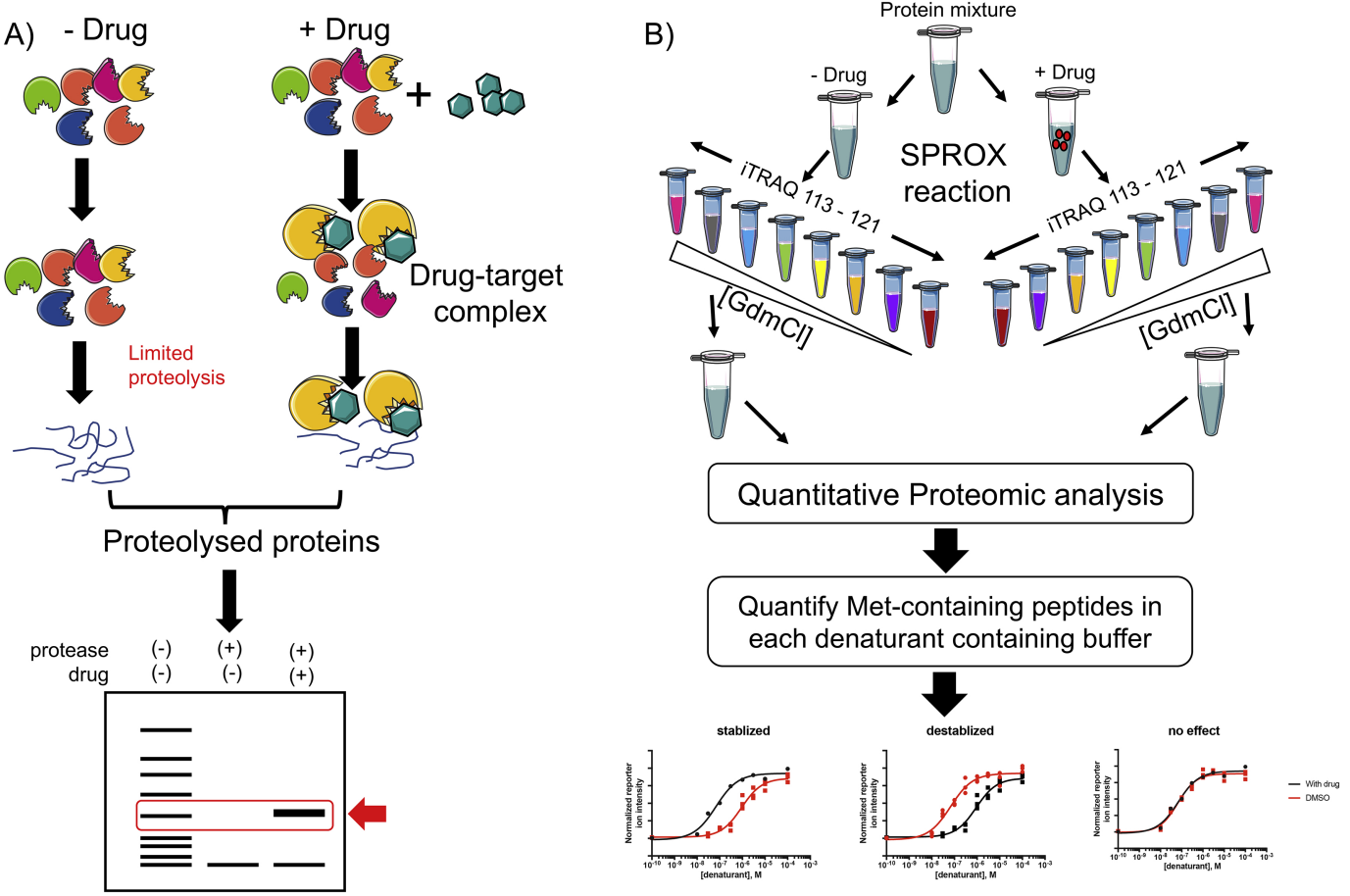
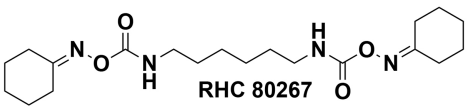
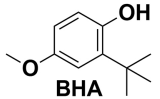
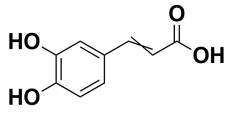
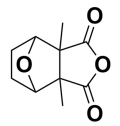

A)
- Drug
+ Drug
Drug-target complex
Limited proteolysis
Proteolysed proteins
protease (-) (+) (+)
drug (-) (-) (+)
B)
Protein mixture
- Drug
+ Drug
SPROX reaction
iTRAQ 113 - 121
iTRAQ 113 - 121
[GdmCl]
[GdmCl]
Quantitative Proteomic analysis
Quantify Met-containing peptides in each denaturant containing buffer
stablized
destablized
no effect
Normalized reporter ion intensity
[denaturant], M
With drug
DMSO

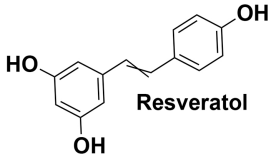
Figure 6

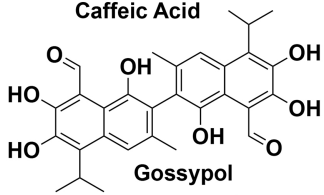

RHC 80267

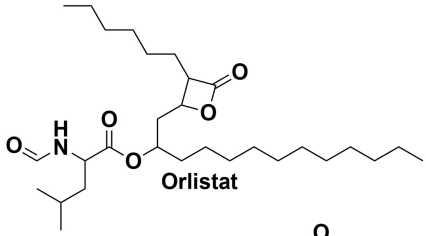

BHA

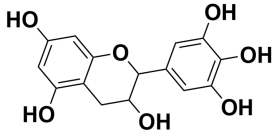

Caffeic Acid

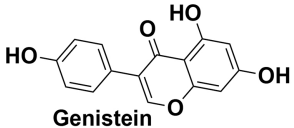

Cantharidin

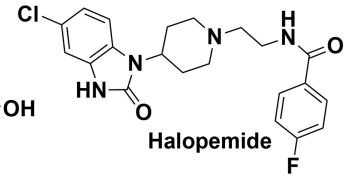

Resveratol

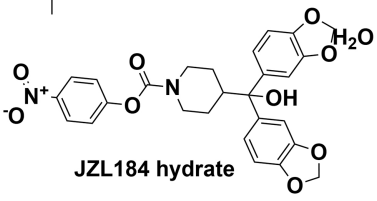

Gossypol

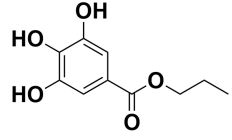

Orlistat

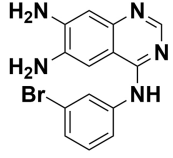

(-)-Epigallocatechin gallate

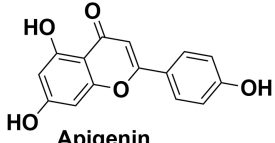

Genistein

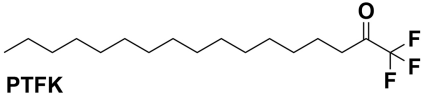

Halopemide

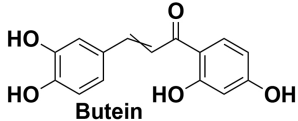

JZL184 hydrate

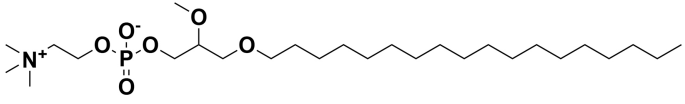

Propyl Gallate


BPDQ


Apigenin


PTFK
(Palmityl trifluromethyl ketone)


Butein


ET-18-OCH3

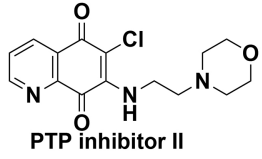
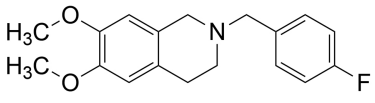

PTP inhibitor II

Figure 7



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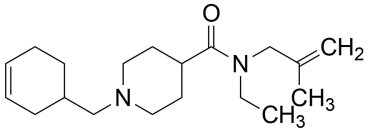

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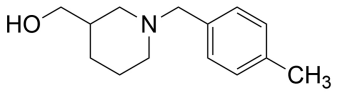

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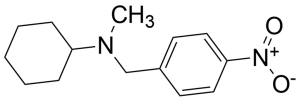
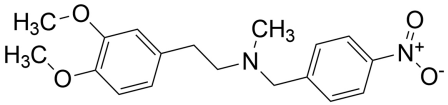


WD30999



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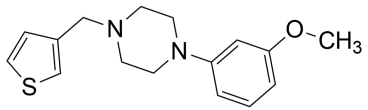
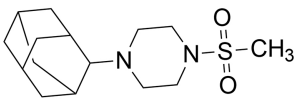
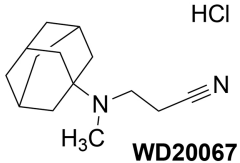


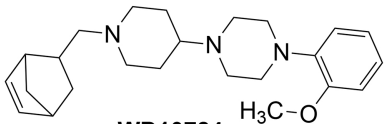
WD10872



WD20542

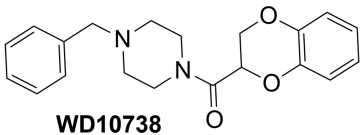


WD20067



WD10784

HCl



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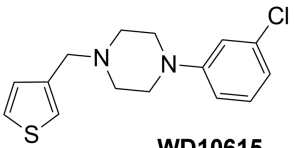
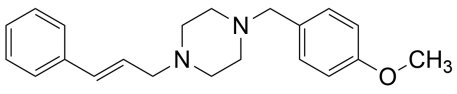
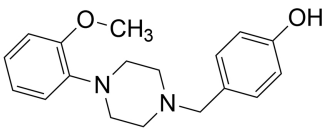


WD10615



WD10599



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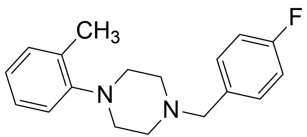

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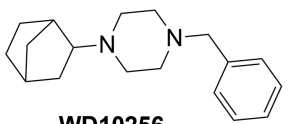

WD10256

Figure 8