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Novel therapeutic drug identification and gene correlation for fatty liver disease using high-content screening: Proof of concept

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a problem in obese people caused by increasing intake of high-calorie food such as fructose implicated in the elevated prevalence. It is necessary to identify novel drugs to develop effective therapies. In this study, we combined LOPAC® (The Library of Pharmacologically Active Compounds) and High-Content screening to identify compounds that significantly reduced intracellular lipid droplets (LD) after high fat medium (HFM) treatment. Among 1280 compounds, we identified 239 compounds that reduced LD by > 50%. Of these, 17 maintained cell viability. Nine of them were selected for validation using normal primary hepatocytes, of which five compounds showed dose-dependent efficacy. Whole genome transcriptomic network analysis was performed to construct the underlying regulatory network. There were 831 (711 up-regulated and 120 down-regulated genes) and 3480 (2009 up-regulated and 1471 down-regulated genes) genes that showed a significant change (> 2-fold; $p < 0.05$) after 12 and 24 h HFM treatment, respectively. Gene enrichment and pathway analysis showed several immune responses mediated by MIF, IL-17, TLR, and IL-6. These compounds modulate lipogenesis via GSK3β and CREB1, which is followed by an alteration in the expression of several downstream genes related to hepatocellular carcinoma and hepatitis. CREB1 is a core transcription factor and may be a potential therapeutic target for liver disease. In conclusion, this proof of concept provides a strategy for identifying novel drugs for treatment of fatty liver disease as well as elucidates their underlying mechanisms. This research provides opportunity for developing future pharmaceutical therapeutics.

1. Introduction

Genetic factors, eating habits, and lifestyle habits cause metabolic syndromes that result in individuals being overweight and obese. Obesity and being overweight are important current problems in the modern population due to lack of exercise and high-calorie diets. In 2016, an estimated 1.9 billion adults were overweight, and of these > 650 million were obese (WHO, 2018). Abnormal or excessive fat accumulation may impair health, with the most common related diseases including cardiovascular disease, type II diabetes, hypertension, and cancer (Collaborators et al., 2017; Lauby-Secretan et al., 2016; McCarthy, 2010). There are various and complex causes of obesity, including genetic predisposition, calorie overloading, and physical inactivity. These factors contribute to energy imbalances and cause body fat heterogeneity, with hypertrophy and hyperplasia of adipose tissue (Karastergiou et al., 2013; McLaughlin et al., 2016; Pellegrinelli et al., 2016). Obesity is associated with an increase in adipose tissue mass, therefore several hormones and enzymes related to lipogenesis are anticipated to have a significant impact on the macronutrient metabolism.

Excess lipid accumulation in the liver leads to hepatitis, steatohepatitis, and fatty liver disease, with either alcoholic or non-alcoholic etiologies. Excessive alcohol consumption is the third most common preventable cause of death in the USA (Mokdad et al., 2004). Chronic or regular alcohol consumption causes several types of liver injury, such as fatty liver, in which hepatocytes contain macrovesicular droplets of triglycerides commonly observed in alcoholic hepatitis. Alcohol-induced liver injury occurs when changes in the NADH-NAD^+ reduction-

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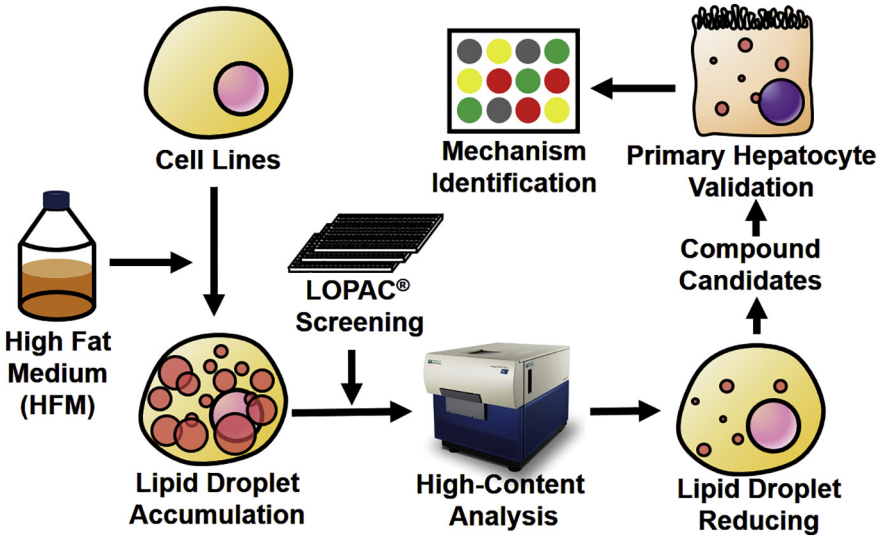

Fig. 1. The experimental design and screening procedure. Hepatocellular carcinoma cells were utilized for the first screening. Cells were treated with HFM and LOPAC® for 16 h followed by intracellular lipid droplet (LD) quantification by High-Content screening. Potential compounds were further validated by mouse primary hepatocyte isolation. Whole transcriptome analysis and a connectivity map were utilized to investigate underlying molecular mechanisms modulated by potential compounds. HFM, high fat medium; LOPAC, Library of Pharmacologically Active Compounds.

oxidation potential inhibit β-oxidation and the tricarboxylic acid cycle, which is followed by lipogenesis promotion (You and Crabb, 2004). Lipid metabolism alterations are enhanced via PPAR-α and AMPK inhibition and SREBP-1 induction (Fischer et al., 2003; Ji et al., 2006; You et al., 2004).

Non-alcoholic fatty liver disease (NAFLD) is currently the most common chronic liver disease in developed countries due to the obesity epidemic. Up to 90% of obese adults are diagnosed with non-alcoholic fatty liver disease (Machado et al., 2006). NAFLD results from increasing uptake of high-calorie food, increased lipogenesis, and lipid oxidation inhibition (Musso et al., 2009), and occurs when the triglyceride content in the liver constitutes > 5% of the liver weight (Kleiner et al., 2005). Recently, evidences showed that fructose over-consumption is suspected to contribute to the increased incidence of NAFLD according to the systematic review (Alwahsh and Gebhardt, 2017; Rebollo et al., 2014). NAFLD can progress to more severe liver diseases such as necrotic inflammation, hepatocyte ballooning, or Mallory body formation, and 10% of NAFLD cases develop into steatohepatitis, liver fibrosis, and liver cancer (Tessari et al., 2009). Recently, a two-hit hypothesis was proposed to explain the causes of NAFLD. The “first hits” are lipid accumulation in the liver and insulin resistance, which sensitize the liver to the “second hits,” which include oxidative stress, abnormal mitochondrial function, hormone interference (leptin and adiponectin), and activated inflammatory cells, causing the disease to progress to hepatitis or fibrosis (Chung et al., 2010; Perez et al., 2003; Yang et al., 1997).

There is no single intervention that efficiently treats both alcoholic and non-alcoholic fatty liver or cures liver disease. For treatment of alcoholic fatty liver, general measures for decompensated liver disease as well as specific measures directed at the underlying liver injury are utilized. Although medications such as corticosteroids, pentoxifylline, anti-TNF-α therapy (Mendenhall et al., 1986), and nutritional support are applied in clinical practice (Akriviadis et al., 2000; Barnes and Karin, 1997), novel drugs for treatment or prevention still have potential benefits for patients. For mild NAFLD patients, regular exercise and calorie control are the best initial treatments (Zelber-Sagi et al., 2007). If these do not work, drugs such as HMG-CoA reductase inhibitors, fibric acid derivatives, and resins are used to decrease serum triglyceride levels (Bakker-Arkema et al., 1996; Fruchart et al., 1998; Zimetbaum et al., 1991). Drugs increasing insulin sensitivity such as Metformin and Thiazolidinediones (TDZs) are also used clinically for NAFLD therapy (Aithal et al., 2008; Promrat et al., 2004). Other agents such as antioxidants, TNF-α inhibitors, IκB kinase inhibitors, caspase inhibitors, and ER stress inhibitors are also potential treatments for NAFLD (Beraza et al., 2008; Kammoun et al., 2009; Lirussi et al., 2007; Witek et al., 2009; Zein et al., 2012). Despite the increasing understanding of the mechanism of NAFLD pathogenesis, few effective liver-specific therapies are available.

In this study, we screened the LOPAC®^{1280} (The Library of Pharmacologically Active Compounds, Sigma-Aldrich) compound library to identify drugs with efficient intracellular lipid droplet (LD) reduction ability using High-Content screening. In this proof of concept investigation, five drugs were identified that effectively dampened lipid accumulation in hepatocytes. This may be useful for hepatosteatosis treatments.

2. Materials and methods

2.1. Cell culture

SK-hep1, HepG2, and Hep3B cells were grown at 37 °C in 5% CO_2-humidified air in a growth medium (GM) composed of DMEM medium (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/ml penicillin (Biological Industries), 100 mg/ml streptomycin (Biological Industries), and 1% L-glutamine (Biological Industries). A total of 2×10^5 cells/well were seeded into six-well plates and incubated in an oil-rich medium for set periods for the Oil-Red O staining. A total of 6×10^3 cells/well were seeded into 96-well plates for fluorescent staining and High-Content screening.

2.2. High fat medium, cholesterol lipid concentrate medium

The high fat medium (HFM) formula was described by De Gotttardi et al. (De Gottardi et al., 2010) and contains DMEM/F12 (Life Technologies, Carlsbad, CA), 10% FBS (Hyclone, Logan, UT), 1% P/S (Biological Industries), ITS (Sigma Aldrich), 0.5 mM palmitic acid (Sigma Aldrich), and 2.05 mM oleic acid (Sigma Aldrich). The 250× Cholesterol Lipid Concentrate (CLC) (Life Technologies, Carlsbad, CA) was diluted to 5× CLC before use.

2.3. LOPAC®^{1280} compound library and drug treatment

In total, 1280 drugs from the LOPAC® compound library (Sigma Aldrich) were dissolved in DMSO to a 1 mM stock and diluted with HFM to 10 μM before cell incubation.

2.4. Oil-Red O staining

Cells treated with HFM or CLC were fixed with 10% formalin for 15 min and washed twice with PBS. Cells were incubated with 60%