REVIEW

Herbal drug discovery for the treatment of nonalcoholic fatty liver disease

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\textbf{Abstract}  Few medications are available for meeting the increasing disease burden of nonalcoholic fatty liver disease (NAFLD) and its progressive stage, nonalcoholic steatohepatitis (NASH). Traditional herbal medicines (THM) have been used for centuries to treat indigenous people with various symptoms but without clarified modern-defined disease types and mechanisms. In modern times, NAFLD was defined as a common chronic disease leading to more studies to understand NAFLD/NASH pathology and progression. THM have garnered increased attention for providing therapeutic candidates for treating NAFLD. In this review, a new model called “multiple organs-multiple hits” is proposed to explain mechanisms of NASH progression. Against this proposed model, the effects and mechanisms of the frequently-studied THM-yielded single anti-NAFLD drug candidates and multiple herb medicines are reviewed, among which silymarin and berberine are already under U.S. FDA-sanctioned phase 4 clinical studies. Furthermore, experimental designs for anti-NAFLD drug discovery from THM in treating NAFLD are discussed. The opportunities and challenges of reverse pharmacology and reverse pharmacokinetic concepts-guided strategies for THM modernization and its global recognition to treat NAFLD are highlighted. Increasing mechanistic evidence is being generated to support the beneficial role of THM in treating NAFLD and anti-NAFLD drug discovery.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is among the most common chronic liver diseases worldwide, and its progressive stage that shows hepatic inflammation and fibrosis is termed as nonalcoholic steatohepatitis (NASH). NASH, in turn can lead to cirrhosis, liver failure and liver cancer. Because of the pandemic spread of obesity, particularly in western countries, the worldwide pooled prevalence of NAFLD continues to increase and is now estimated at 24%. Notably, 8%–19% of NAFLD patients are found to be lean or non-obese in Asia. NAFLD has become the second leading cause of liver transplantation in the United States.

Generally, the non-progressive stage of NAFLD is asymptomatic and pharmacologically curable, while the progressive NASH is refractory to treatment. Most market-available drugs, such as vitamin E, only improve hepatic steatosis and inflammation, but have little impact on the progressive fibrosis, during treatment of NAFLD. Diverse clinical trials for testing modern drug candidates of NASH have failed to reach the major endpoint or has limited therapy efficacy, such as obeticholic acid. Several agents such as nuclear receptor agonists (obeticholic acid, GFT505, elafibranor), insulin sensitizers (glitazones, pioglitazone, metformin) and glucagon-like peptide-1 receptor agonists are still in the drug pipeline for NASH. It takes up to three years for obtaining outcomes to register promising anti-NASH drugs, and no drugs have been approved by the U.S. Food and Drug Administration (FDA) to treat NASH until now. Currently only weight loss by bariatric surgery treatment or non-pharmacological managements by healthy life/diet style and/or physical activity can be effective. Thus, the development of medicines for treating NAFLD, especially the incurable NASH, is an unmet medical need.

Traditional herb medicines (THM), a predominant source of natural medicines and herbal products, are indispensable sources for developing hepatoprotective drugs. Although there is still no compelling evidence from large-scale randomized controlled trails (RCTs) to support the therapeutic effects of THM, a recent survey showed that 20%–30% of patients used traditional medicine in Indonesia for treating various diseases and THM use in some Asian countries has increased in recent years. A survey showed a similar percentage of herbal use for treating chronic liver disease as complementary and alternative medicine. In another systematic meta-analysis, traditional Chinese medicine (TCM) decreases alanine aminotransferase (ALT), aspartate aminotransferase (AST) and radiological steatosis and thus benefits the treatment of NAFLD, suggesting TCM have modest benefits in the treatment of NAFLD in 62 RCTs among 25,661 patients from 419 clinical studies. Thus, prior to the development of conclusive evidence-based effective pharmacological therapies, the clinical use of THM plays a non-negligible role in treating NASH. The expanding knowledge of THM in benefiting the improvement of metabolic diseases, especially NAFLD and NASH, against the extremely long period of modern drug discovery, has driven studies to pursue the potential efficacious and safe therapies by use of THM, which could be called “a natural combinatorial chemical sample library” gift from ancient practical experiences.

In this review, to better elucidate how THM provides NAFLD/NASH improvement and anti-NASH drug discovery, we first summarized the FDA-sanctioned clinical studies of herbal products. To better understand the effects and mechanisms of THM in anti-NAFLD or drug discovery, we firstly reviewed the updated publications about NAFLD/NASH pathogenesis, and we proposed a new “multiple organs-multiple hits” model for updating the explanation of NAFLD/NASH progression mechanisms. Against the proposed “multiple organs-multiple hits” NAFLD/NASH progression model, the emerging effects and molecular mechanisms/targets for the frequently-studied herbs are reviewed. To benefit the discovery of herb-derived anti-NAFLD drugs, the reverse-pharmacology and reverse-pharmacokinetic concepts for guiding preclinical experimental design, as well as experiment design details including NAFLD model choice, drug dosing method choice, and new technology-derived omics/hypothesis-based mechanism exploration for studying the effects of THM on NAFLD are discussed. This review will update the understanding of NAFLD/NASH pathogenesis mechanisms, effects and mechanisms of anti-NAFLD herbs, and guides the discovery of anti-NAFLD drugs from traditional herbs.

2. THM in this era: source and market, clinical trials, preclinical studies

2.1. THM source, market and modernization

THM, used to treat illness that could date back more than 5000 years, are mainly sourced from TCM, Ayurveda, Japanese and Kampo medicine. They have been used and recorded in their respective ancient medical books by the prior civilizations in countries including such as China, India, Japan, Arabia. To promote THM modernization, a plan by the Chinese government was launched in 2007 to expand basic and clinical research on TCM. TCM modernization has achieved significant progress as revealed by the fact that most FDA-sanctioned clinical trials are on TCM-derived drugs (Table 1). However, the modern knowledge for how best to use THM is still limited and no conclusive clinical results are found to support the therapeutic potential of TCM. Further, against the common belief that “natural is safe and better”, some THM have been associated with toxicity that indicates a potential risk for use of THM. Thus, more efforts are still needed to fully clarify the mechanisms and targets of THM-derived compounds, including detailed hepatoprotective effects and research strategies, for guiding patients or consumers to better use THM.
2.2. THM clinical trials

Although not approved by the FDA, many THM have already been used in the clinic for treating various types of liver diseases including NAFLD/NASH. In this era of “precision medicine” and “evidence-based medicine”, developing large-scale RCTs is extremely important and urgently needed to conclusively validate whether a given THM is effective in NAFLD or NASH treatment. A search of ClinicalTrials.gov lists 737 studies for treating NAFLD, among which 25 are traditional herbs (Table 1). More than 20 out of these 25 herbs include or belong to TCM. Seven studies are silymarin related, five for resveratrol, three for curcumin, while two are for berberine.

Among these frequently studied THM, silymarin contains multiple active compounds, while resveratrol, curcumin and berberine are single compounds. Notably, both silymarin and berberine have been registered as phase 4 clinical trials for treating NAFLD or NASH. Limited studies are on ginger and Zhengzhu Tiaozi Capsule. Eight of 25 are used as drugs, while another 17 are used as dietary supplements. As an unmet therapeutic need, anti-NASH drugs have been granted an accelerated access pathway to be approved by the FDA34. The results and clinical trial outcomes are expected to come within 1−3 years for these two promising compounds.

2.3. Hepatoprotective effects and mechanisms of THM in treating NAFLD and NASH

2.3.1. A “multiple organs-multiple hits” model for updating NAFLD pathogenesis and pharmacotherapy targets

Many recent publications on molecular NAFLD-promoting pathways have updated the original “two-hit” theory (the first hit depends on steatosis and the second hit from other pathogenic factors such as oxidative stress and inflammation), as recently reviewed1,35-42. Multiple targets by combined coordination among different organs is known to drive hepatic NAFLD progression43. Thus, in this review, by combining previously-proposed NAFLD progression models with recently-updated research results44-48, a “multiple organs-multiple hits” model is proposed to explain NASH pathogenesis and pharmacotherapy targets (Fig. 1). In this “multiple organs-multiple hits” model, the crosstalk between liver and multiple organs (such as gut, white/brown adipose tissue, skeletal muscle and central nervous system) is described. On one hand, in the liver, free fatty acids from either lipolysis of white

| Table 1 Clinical trials for THM in treating NAFLD/NASH registered at ClinicalTrials.gov. |
| Study title | Status | Identifier |
| Role of pioglitazone and berberine in treatment of NAFLD | Completed, phase 2 | NCT00633282 |
| Efficacy and safety of berberine in NASH22-25 | Recruiting, phase 4 | NCT03198572 |
| Silymarin in NAFLD | Not recruiting, phase 4 | NCT02973295 |
| Silymarin for the treatment of NAFLD36 | Completed, phase 2 | NCT02006498 |
| Effect of silymarin in patients with NAFLD | Recruiting, N/A | NCT03749070 |
| Phase I trial of silymarin for chronic liver diseases | Completed, phase 1 | NCT00389376 |
| Study to evaluate the effect of RGMAM001 on patients with NAFLD | Unknown, N/A | NCT01511523 |
| Phase II trial of silymarin for non-cirrhotic patients with NASH | Completed, phase 2 | NCT00680407 |
| Efficacy of a natural components mixture in the treatment of NAFLD | Completed, N/A | NCT02369536 |
| Resveratrol for the treatment of NAFLD and insulin resistance in overweight adolescents27 | Completed, phase 2/3 | NCT02216552 |
| Long-term investigation of resveratrol on fat metabolism in obese men with NAFLD38,39 | Completed, N/A | NCT01446276 |
| The effects of resveratrol supplement on biochemical factors and hepatic fibrosis in patients with NASH4 | Completed, phase 2/3 | NCT02030977 |
| Resveratrol in patients with NAFLD | Completed, N/A | NCT01464801 |
| Potential beneficial effects of Resveratrol | Completed, N/A | NCT01150955 |
| Therapeutic effects of compound Zhengzhu Tiaozi capsules in NAFLD | Recruiting, N/A | NCT03375580 |
| Purified anthocyanin and NAFLD32 | Completed, phase 1 | NCT01940263 |
| Clinical investigation on the effects of bayberry juice treatment in adult subjects with features of fatty liver disease24 | Completed, N/A | NCT01707914 |
| The clinical trial of NAFLD treated by TCM | Completed, phase 1 | NCT01677325 |
| Curcumin supplement in NAFL patients with type 2 diabetes | Completed, phase 2/3 | NCT02908152 |
| The effects of Zataria Multiflora Boiss. (Shirazi’s thyme) on NAFLD | Completed, N/A | NCT02983669 |
| Effect of ginger supplement on NAFL | Completed, phase 2/3 | NCT02535195 |
| Effects of ginger on NAFLD in T2DM | Enrolling, phase 1 | NCT02289235 |
| The effect of Protandim on NASH | Completed, N/A | NCT00977730 |
| A study of Siliphos in adults with NASH32 | Completed, phase 2 | NCT00443079 |
| The effect of curcumin on liver fat content in obese subjects | Not recruiting, N/A | NCT03864783 |

A search of key word “NAFLD” or “NASH” in the item “Condition or disease” at ClinicalTrials.gov (at July 22, 2019) yielded 737 listed studies, 25 of which were found and listed in this table that are related to traditional herbs.

NAFL, non-alcoholic fatty liver; T2DM, type 2 diabetes mellitus.
N/A, not available.
adipose tissue or hepatic lipid droplets or de novo lipogenesis from dietary fats/sugars are overloaded. Then, fatty acids could be removed by fatty acid β-oxidation (regulated by peroxisome proliferator-activated receptor alpha (PPARA)-activation) or formation of lipotoxic lipids or triglycerides. When the disposal pathways of over-loaded fatty acids are saturated, fatty acids then form lipotoxic species that could cause oxidative stress, ER stress, inflammation and possibly cell death. Non-liver organs can also contribute directly or indirectly to NASH progression. All the pathogen-associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) generated from fatty acids-overloaded liver, "leaky gut" or inflamed macrophages, together work to activate hepatic stellate cells (HSC) and cause liver fibrosis.

2.3.1.1. Gut—liver axis in NAFLD/NASH. Intestine factors including both host and microbiota play a key role in mediating NASH progression via the gut—liver axis as reviewed previously. Many intestinal mediators produced from either host or microbiota could act as bioactive signaling molecules, PAMPs or DAMPs that are circulated via the portal vein. All the PAMPs and/or DAMPs generated from liver, gut or macrophages can work together to activate HSC and cause liver fibrosis. Energy balance could be regulated by the central nervous system via food intake or central hormones/signaling, as well as by brown adipose or skeletal muscle that helps burn adipose fat via thermogenesis or energy expenditure, thus indirectly decrease the overloaded burden of hepatic fatty acids and alleviate NAFLD.

Figure 1  Proposed “multiple organs-multiple hits” model for explaining the NAFLD/NASH pathogenesis. “Multiple organs-multiple hits” model describes how the crosstalk between liver and other tissues/non-liver cells (gut, brown/white adipose, skeletal muscle, brain and macrophages) promotes NASH progression. Free fatty acids, sourced from either adipose lipolysis or hepatic lipid droplets or de novo lipogenesis from dietary fats/sugars, overload the liver. Then, fatty acids could be degraded by fatty acid β-oxidation and triglyceride secreted from liver to serum, or can be converted to lipotoxic lipids when the disposal pathways of fatty acids are saturated. Lipotoxic lipids could then cause oxidative stress, ER stress, inflammation and possibly cell death. Non-liver organs can also contribute directly or indirectly to NASH progression. During NAFLD progress, changes in gut microbiota composition or intestinal lipid modulation signaling, can yield toxic microbiota products, or even forms leaky gut to release LPS or bacteria, all of which could be PAMPs or DAMPs and enter the liver via the portal vein. All the PAMPs and/or DAMPs generated from liver, gut or macrophages can work together to activate HSC and cause liver fibrosis. Energy balance could be regulated by the central nervous system via food intake or central hormones/signaling, as well as by brown adipose or skeletal muscle that helps burn adipose fat via thermogenesis or energy expenditure, thus indirectly decrease the overloaded burden of hepatic fatty acids and alleviate NAFLD.
Role of traditional herbs in nonalcoholic fatty liver disease

(HFD) feeding induces activation of intestinal hypoxia-inducible factor 2 alpha (HIF2A) that contributes to hepatic steatosis via directly modulating intestinal ceramide metabolism to reduce the ceramide levels43. Numerous studies have revealed the role of microbiota in mediating NAFLD progression, exemplified by the fact that gut microbiota composition was altered in obese rodent animals or human patients46,53, and probiotics used to normalize the microbiota composition, could therapeutically alleviate NAFLD progression in experimental animal models49,55. In addition, increased intestinal permeability (leaky gut) increases exposure of the liver to gut or microbiota-derived bacterial products such as lipopolysaccharides (LPS) that could act as PAMPs or DAMPs to stimulate innate immune systems such as toll-like receptors (TLRs) and NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation56,57.

2.3.1.2. Immune system in NAFLD/NASH. NAFLD is characterized by the hallmarks of inflammation, hepatocyte death and the resulting fibrosis. Obesity, a major cause of NAFLD, is associated with low-grade systemic inflammation through disturbance of the immune system54. The key immune cells including Kupffer cells, dendritic cells, neutrophils, and natural killer cells could promote NAFLD/NASH progression by sensing DAMPs released from damaged hepatocytes or PAMPs derived from other tissues such as intestine (bacterial-produced LPS or other toxins) or adipose tissue (adipokine imbalance)55. The role of Kupffer cells in regulating the progression of NAFLD is well-established, although the role of other immune cells in NAFLD/NASH development is still controversial. Depletion of Kupffer cells improves NAFLD/NASH, supporting a pro-NASH role of Kupffer cells58, while further studies revealed the activated M1 phenotype as pro-inflammatory promotion of NAFLD/NASH progression and the M2 as immunoregulatory that inhibits NAFLD/NASH progression55,57. Thus, innate immune system modulation presents an opportunity for NASH therapy. Notably, immune cells also directly provide novel molecular anti-NASH targets. For example, immune cell-expressed TLRs could sense PAMPs and DAMPs that could mediate NAFLD/NASH progression via PAMPs/DAMPs-TLR-myeloid differentiation primary response 88 (Myd88) pathway41. Immune cells also, at least partially, contribute to NLRP3 inflammasome activation that may mediate NAFLD progression46,52, while NLRP3 inflammasome blockade by NLRP3 inhibitor reduces experimental NASH in mice56, suggesting that NLRP3 inflammasome pathway is an effective druggable target for NASH pharmacotherapy.

2.3.1.3. Adipose and skeletal muscle in NAFLD/NASH. Brown adipose tissue or subcutaneous white adipose tissue “browning” or skeletal muscle could help burn fat via uncoupling protein 1 (UCP1)-dependent thermogenesis or PPARG coactivator 1 alpha (PGC1A)-dependent energy expenditure47,60. White adipose tissue could mediate adipose lipolysis (mainly via hormone-sensitive lipase, HSL)61 and mediate UCP1-dependent independent thermogenesis62. Many genes specifically expressed in adipose or skeletal muscle have the potential to mediate NAFLD development or NASH progression via modulating obesity. For example, adipose-specific SIRT6 ablation sensitizes mice to HFD-induced obesity and hepatic steatosis by inhibiting lipolysis63.

2.3.1.4. Central nervous system in NAFLD/NASH. Central nervous system mediates metabolic syndrome and hepatic lipid/lipoprotein metabolism via various mechanisms64. For example, the central nervous system modulates food intake (appetite)65, regulates gut-nervous system axis66,67, senses glucagon like peptide-1 (GLP-1)68, and mediates endogenous hormones (such as leptin and insulin) secretion69. For example, central nervous system regulates hepatic lipogenesis, lipid oxidation of brown adipose tissue and thus modulates energy balance via central actions of thyroid hormones through pathway of hypothalamic AMP-activated protein kinase (AMPK)-ER stress-c-Jun N-terminal kinase 1 axis69. Genetic ablation of activating transcription factor 4 and/or 5 in hypothalamic proopiomelanocortin neuron modulates energy expenditure and thus HFD-induced obesity and obesity-associated fatty liver70.

2.3.2. Hepatoprotective effects and mechanisms of traditional herbs in treating NAFLD/NASH

Herbal drug(s) (combination therapies or single drugs) that target one or multiple hits of the “multiple organs-multiple hits” pathogenesis processes could affect NASH. The structures (Fig. 2) and possible molecular hepatoprotective mechanisms (Table 271-122) of the most frequently investigated THM for treating NAFLD and NASH and their are listed below.

2.3.2.1. Resveratrol. Resveratrol, a natural polyphenol that activates sirtuin 1 (SIRT1), has demonstrated potent effects in alleviating NAFLD in both rodent and cell models, while its effect in NAFLD patients is still inconclusive123. A clinical study in 24 obese but otherwise healthy men showed that a high dose of resveratrol did not affect body composition, insulin sensitivity, and other inflammatory or metabolic biomarkers124, while other studies in non-obese middle-aged subjects with normal glucose tolerance also failed to benefit from resveratrol supplements125, suggesting that resveratrol has no efficacy in humans that do not have metabolic disease. In contrast, for metabolic disease patients, a study of 50 NAFLD patients revealed that 500 mg of resveratrol treatment for 12 weeks could further benefit NAFLD treatment when compared to lifestyle modification alone, partially by inhibiting inflammation and apoptotic liver injury31. However, other studies revealed no benefit of resveratrol in metabolic disease patients. For example, in one clinical study with 16 obese male NAFLD patients, resveratrol showed no significant metabolic benefit126. Similarly, an updated systematic review and meta-analysis for four RCTs (n = 158 patients) concluded that resveratrol supplementation in NAFLD patients had no efficacy in attenuating NAFLD126. Thus, evidence for the clinical benefit of resveratrol in treating NAFLD is still inconclusive, albeit many studies demonstrated the effect of resveratrol in treating NAFLD in rodent models.

Mechanically, resveratrol activates AMPK74,76 and/or SIRT1 signaling to suppress lipogenesis in liver77-79 or induces white adipose browning via activating AMPK80. Resveratrol inhibits the NLRP3 inflammasome activation to attenuate hepatic metainflammation81; and modulates autophagy and NFkappaB1 activity in diet-induced NAFLD murine models82,83. Resveratrol also inhibits liver X receptor alpha (LXR)-dependent hepatic lipogenesis through antioxidant activity84. Although no direct report showed how nuclear factor erythroid 2-related factor 2 (NRF2) activation mediates the hepatoprotective role of resveratrol in treating NAFLD, resveratrol was demonstrated to activate NRF2 and thus protect cells from oxidative stress both in vivo and in vitro, dependent on the presence of NRF282,83.
Notably, resveratrol is a poorly-absorbed polyphenol and is metabolized rapidly, so it could be mainly detected in the small intestine after gavage for 1 h in rats\textsuperscript{127}. Consistent with its predominant distribution in the intestines when dosed by gavage, resveratrol was found to target the duodenal AMPK–SIRT1 axis and vagal gut–brain neuronal axis to reverse the metabolic syndrome in obese and diabetic rats\textsuperscript{84}. In addition, resveratrol is partly metabolized by gut microbiota to produce resveratrol derivatives that could have similar biological effects with resveratrol, and resveratrol changes the composition of gut microbiota to yield beneficial metabolic outcomes\textsuperscript{85,86}. Resveratrol improves the HFD-induced gut microbiota dysbiosis by increasing the Bacteroidetes-to-Firmicutes ratios, significantly inhibiting the growth of Enterococcus faecalis, and increasing the growth of Lactobacillus and Bifidobacterium\textsuperscript{87}. Another study further demonstrates that resveratrol could be metabolized by the human gut microbiota to yield dihydroresveratrol, 3,4'-dihydroxy-trans-stilbene and 3,4'-dihydroxybibenzyl lunularin; and two strains, Slackia equolifaciens and Adlercreutzia equolifaciens, were identified as dihydroresveratrol producers\textsuperscript{88}. Resveratrol also maintains junctions between intestinal cells, contributes to gut barrier integrity and could prevent “leaky gut”\textsuperscript{86}. Thus, resveratrol activates NRF2 signaling and both hepatic and intestinal SIRT1 and AMPK signaling, modulates cell death and regulates gut microbiota and intestine integrity that could affect NAFLD. However, the translation of its effect from mouse models to humans remains uncertain.

2.3.2.2. Curcumin. Curcumin, a natural polyphenol, is the major active compound of turmeric and has been historically used in TCM or Ayurvedic medicine. Preclinical murine models demonstrate that curcumin attenuates NAFLD\textsuperscript{128} and clinical trials demonstrate curcumin as a promising, but not proven, treatment for NAFLD\textsuperscript{129}. Curcumin is currently in phase II/III clinical trials (Table 1). However, the clinical benefit for curcumin in treating NAFLD is still uncertain. In a trial of 80 patients in Iran, curcumin significantly improved fatty liver (78.9% by curcumin vs. 27.5% by placebo) and other NAFLD parameters including aminotransferases\textsuperscript{130}. Extensive preclinical studies support the hepatoprotective role of curcumin in treating NAFLD and NASH. Curcumin attenuates NFKB1 activation and improves methionine-choline-deficient (MCD) diet-induced NASH in mice\textsuperscript{89}, HFD-induced NASH in rabbits\textsuperscript{90}, and prevents fatty liver in fructose-fed rats\textsuperscript{91}. Curcumin also retards the NASH progression in a NASH-hepatocarcinoma cancer mouse model\textsuperscript{131}. Mechanically, curcumin improves NASH by inhibiting apoptosis and protecting mitochondria in HFD-induced NAFLD in rats\textsuperscript{92}, and limits the fibrogenic evolution of MCD diet-induced steatohepatitis \textit{in vivo}. 

Figure 2  Chemical structures of frequently-examined THM-derived components for the treatment of NAFLD in clinical trials registered at Clinicaltrials.gov. Compounds A–G are hepatoprotective components isolated from silymarin, while curcumin (H), resveratrol (I) and berberine (J) belong to single compound drug.
in mice\textsuperscript{97}, and attenuated HFD-induced hepatic steatosis by activating AMPK and increasing PPARA signaling\textsuperscript{99}. Curcumin also decreased oleic acid-induced lipid accumulation via AMPK phosphorylation \textit{in vitro} in hepatocarcinoma cells\textsuperscript{98}. By using Nlrp3-deficient mice, curcumin was confirmed to inhibit NLRP3 inflammasome activation and suppress interleukin 1B (IL1B) secretion and inflammation in HFD-fed mice depending on the presence of NLRP3\textsuperscript{100}.

Curcumin repressed NLRP3 inflammasome activation \textit{via} the TLR4/Myd88/NFkB1 and P2x purinoceptor 7 (P2X7) signaling in macrophages\textsuperscript{101}. It protects against LPS-induced septic shock \textit{via} suppressing NLRP3 inflammasome activation\textsuperscript{102}. Curcumin also alleviated HFD-induced metabolic endotoxemia and intestinal inflammation and attenuated hepatic steatosis in rats \textit{via} modulation of gut microbiota\textsuperscript{103}. Thus, curcumin decreases NAFLD \textit{via} activating AMPK, Nrf2 and nuclear receptors to modulate lipid metabolism and oxidative stress, while it also inhibits NLRP3 inflammasome activation and gut microbiota to modulate inflammation and decrease hepatic steatosis, but its effect in clinical NAFLD patients still needs further validation.

### Table 2

| Herb          | Molecular pathway                                                                 |
|---------------|-----------------------------------------------------------------------------------|
| Resveratrol   | SIRT1 activator\textsuperscript{1,73}, AMPK activator\textsuperscript{4,76}       |
|               | Adipose AMPK activation to induce white adipose browning\textsuperscript{7}       |
|               | NLRP3 antagonism\textsuperscript{78}                                               |
|               | Autophagy induction and NFKB1 activation\textsuperscript{79,80}                    |
|               | Inhibition of LXR-dependent hepatic lipogenesis\textsuperscript{84}               |
|               | NRF2 activator\textsuperscript{83}                                                |
|               | Gut AMPK-SIRT1 axis\textsuperscript{84}                                            |
|               | Gut–brain neuronal axis\textsuperscript{84}                                        |
|               | Modulating gut microbiota composition\textsuperscript{85–87}                      |
|               | Gut microbiota-derived metabolites\textsuperscript{88}                             |
|               | Protecting “leaky gut”\textsuperscript{89}                                        |
|               | NFKB1 inhibition\textsuperscript{90}                                              |
|               | Inhibiting apoptosis\textsuperscript{92}                                           |
|               | Reducing TIMP1 secretion\textsuperscript{93}                                      |
|               | PPPAR activation\textsuperscript{94}                                              |
|               | Interrupting leptin signaling\textsuperscript{95,96}                              |
|               | Activating Nrf2, Fxr and Lxr\textsuperscript{97}                                  |
|               | AMPK activation\textsuperscript{98}                                                |
|               | and Pparg activation\textsuperscript{99}                                          |
|               | NLRP3-dependent anti-inflammation\textsuperscript{100}                            |
|               | TLR4–MyD88 axis\textsuperscript{101}                                               |
|               | Gut microbiota modulation\textsuperscript{102,103}                                |
|               | Activate adipose thermogenesis\textsuperscript{104}                               |
|               | Inhibition of NLRP3 and ER stress and autophagy induction\textsuperscript{105,106}|
|               | Nrf2 and Ppar activation\textsuperscript{107–109}                                |
|               | Systematic AMPK activation\textsuperscript{104,110,111}                           |
|               | Intestinal Fxr signaling and bile acids modulation\textsuperscript{112}           |
|               | Modulating gut microbiota\textsuperscript{113,114}                                |
|               | Producing microbiota-derived metabolites\textsuperscript{115,117}                 |
| Silymarin     | Sirt1/AMPK activation\textsuperscript{118}                                        |
|               | Fxr and AMPK activation\textsuperscript{119,120}                                  |
|               | NLRP3 signaling antagonism\textsuperscript{121}                                   |
|               | Antioxidant that could increase Nrf2 translocation\textsuperscript{121,122}      |

### 2.3.2.3. Berberine

Berberine is a natural alkaloid present in \textit{Coptis Chinensis} and several other Chinese herbal medicines. Although most commonly used for treating diarrhea in China, extensive experimental studies demonstrated efficacy of berberine as a hypolipidemic and NAFLD protectant both in various rodent NAFLD/NASH animal models and NAFLD/NASH patients\textsuperscript{133,134}. In one trial among 184 NAFLD patients from three medical centers, berberine plus lifestyle intervention resulted in a significant NAFLD improvement compared with lifestyle intervention alone\textsuperscript{24}. In another trial on 80 patients, berberine treatment markedly decreased serum levels of lipid species and ceramides\textsuperscript{35}. Due to a limitation in the number and quality of clinical trials, further efficacy validation of berberine on NAFLD patients is warranted\textsuperscript{136}. A phase 4 clinical trial (ClinicalTrials.gov Identifier: NCT03198572) of berberine was launched for treating NASH\textsuperscript{(Table 1)}. Many mechanisms were suggested to explain how berberine might alleviate NAFLD. Berberine improves oxidative stress by activating thermogenesis in both brown adipose tissue and white adipose tissue\textsuperscript{104}. Berberine was found to improve NAFLD/NASH by suppressing ER stress, NLRP3 inflammasome activation and liver inflammation in genetically-obese db/db mice, MCD diet-fed mice or in \textit{ApoE}\textsuperscript{−/−} mice\textsuperscript{26,105,106,137,138}. Berberine inhibited NLRP3 inflammasome activation \textit{in vitro} and \textit{in vivo} in two unrelated murine models, MCD-induced NASH and acetaminophen-induced acute liver injury, and modulating P2x7 signaling to antagonize NLRP3 inflammasome activation in LPS-treated macrophages \textit{in vitro}\textsuperscript{106}. Berberine also attenuated palmitate-induced NLRP3 inflammasome activation \textit{via} triggering autophagy in macrophages \textit{in vitro} and attenuated HFD-induced insulin resistance \textit{in vivo}\textsuperscript{103}. Berberine is known to activate Nrf2 signaling and Ppar activation, and suppress oxidative stress-related liver injury in rats\textsuperscript{107}, while Nrf2 activation by berberine is also found in macrophage\textsuperscript{108}. Berberine ameliorates fatty acid-induced oxidative stress in both HFD-fed mice and fatty acid-treated human hepatoma cells\textsuperscript{109}. However, there is still no direct evidence to demonstrate a direct role for berberine in Nrf2 as well as directly reduces tissue inhibitor of metalloproteinase 1 (TIMP1) secretion and oxidative stress in HSC \textit{in vitro}\textsuperscript{75}. Curcumin inhibits expression of receptors for advanced glycation end-products in HCS \textit{in vitro} by inducing Ppar activation and inhibiting oxidative stress\textsuperscript{82}. In addition, other studies found that curcumin eliminated the effect of advanced glycation end-products by interrupting leptin signaling\textsuperscript{83}, and attenuated the effects of insulin on stimulating HSC activation \textit{via} affecting insulin signaling and oxidative stress\textsuperscript{84}. Curcumin activated the Nrf2, Fxr and Lxr signaling pathways to regulate metabolism and attenuate NAFLD.

### Summary of possible molecular pathways that are involved in the hepatoprotective effects for resveratrol, curcumin, berberine, and silymarin.

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activation in vitro with NRF2-driven luciferase activity. In addition, berberine activation of central, peripheral and adipose AMPK may be of value in treating diabetes and fatty liver.

Interestingly, berberine, known to have extremely low bioavailability (<1%), was mainly distributed in intestines after gavage and was reported to modulate hepatic lipid metabolism by directly modulating gut microbiota and microbial bile acid metabolism through intestinal FXR signaling. Berberine increased short-chain fatty acid-producing bacteria including Allobaculum, Bacteroides, Blautia, Butyricoccus, and Phascolarctobacterium in HFD-fed rats. Berberine could be metabolized by the gut microbiota in a form that is more rapidly absorbed in the intestine and thus nitroreductases, encoded by intestinal bacteria, could promote intestinal absorption of berberine. Berberine could induce the production of gut microbiota-derived bioactive metabolites such as butyrate and thus improve energy metabolism. Thus, berberine targets multiple molecular hepatoprotective mechanisms related with organs including liver, macrophage, gut, microbiota and adipose. Its translational effect in treating NASH is in a phase 4 clinical trials.

2.3.2.4. Silymarin. Silymarin, also known as milk thistle extract, has mixed components that show potent hepatoprotective effects in various types of liver diseases. As multiple drug mixtures, the effective components include taxifolin, isosilybin A, silybin A, silybin B, silybinin, or their mixed forms (Fig. 2). Preclinically, silymarin or its hepatoprotective component(s) were demonstrated to alleviate NAFLD in MCD diet-fed C57BL/6J mice and in db/db mice, and in HFD-induced NAFLD in hamsters and mice. Oral administration of silymarin improved a juvenile model of NASH. A clinical trial with 64 NASH patients registered in Iran demonstrated that silymarin significantly decreased serum ALT and AST. Similarly, several other clinical studies indicate that silymarin could improve NAFLD. However, other clinical studies also indicate that the effect of silymarin in treating NAFLD or NASH is limited. One trial with 99 NASH patients demonstrated that silymarin only reduced liver fibrosis and reduced NAFLD activity scores less than 30%. A systematic meta-analysis of 10,904 publications suggested that silymarin only minimally reduces ALT and AST without clinical relevance. Thus, the data on silymarin clinical outcomes are still controversial. To further establish the clinical effects of silymarin in treating NAFLD, a phase 4 clinical study (ClinicalTrials.gov Identifier: NCT02973295) was launched for evaluating the effect of silymarin in treating NAFLD (Table 1) and the results outcome is expected to be obtained within 1–3 years.

The controversial data of silymarin clinical outcomes may result from patient variance and different dosing regimens. However, it is still generally believed that all the three main silymarin-contained flavonoids, silybin, silydianin and silychristin, have poor bioavailability, which might be the cause of its compromised hepatoprotective effect in clinical patients. Indeed, some studies have tried to improve the bioavailability of silymarin by using several methods. For example, phytosomes (silybin–phosphatidylcholine complexed lipid-compatible molecules) were tested to enhance bioavailability. A clinical study revealed that the efficacy of a silybin/vitamin E/phospholipid complex improved insulin resistance and NAFLD. The beneficial effect of this complex in NAFLD patients was further confirmed in another multicenter trial.

A mechanism was suggested that silibin restored NAD+ levels to induce the SIRT1/AMPK pathway in HFD-fed mice and palmitate-treated HepG2 cells. Silymarin or silibin also activated FXR signaling or AMPK phosphorylation to modulate lipid metabolism in HFD-fed rodents. Silybin alleviated NLRP3 inflammasome assembly via the NAD+/SIRT2 pathway in a rodent NAFLD model. Silymarin is also a known antioxidant, which could increase NRF2 translocation in MCD diet-fed mouse livers. Although several other polyphenols such as quercetin and naringenin were demonstrated to modulate the gut microbiota, to the best of our knowledge, there are no studies investigating how silymarin affects the gut microbiota. As one type of several mixed polyphenols, silymarin has a potential role in altering intestinal microbiota that may in turn mediate its effects on metabolic diseases. Thus, silymarin affects multiple targets, including FXR, NRF2, SIRT1, SIRT2, AMPK and NLRP3 inflammasome to restore NAFLD or NASH. Clinical results indicate promising, but still not conclusive, role for silymarin in treating NAFLD or NASH.

2.3.2.5. Silymarin-contained mixtures. Silymarin is also used as an important ingredient for complex mixtures. For example, one nutraceutical mixture contains ingredients of natural origin, including fish oil, 70% docosahexaenoic acid, phosphatidylcholine, silymarin, choline bitartrate, curcumin, alpha-tocopherol and choline. This mixture was registered in ClinicalTrials.gov (NCT02369536) for treating NAFLD (Table 1), but no results have been posted. Another example is siliphos, a silibinin-phosphatidylcholine complex. The main purpose of forming silybin-phosphatidylcholine complex is to facilitate the transmembrane ability of silybin and improve the absorption of this poorly absorbed flavonoid to enhance the hepatoprotective effect. Siliphos prevented mitochondrial dysfunction, oxidative stress, inflammation and liver fibrosis in a rodent model of NASH.

2.3.2.6. THM beyond the registration. Other THM, although clinical trials not currently registered in ClinicalTrials.gov for treating NAFLD and NASH, showed potential for treating NAFLD in preclinical studies. With some single compounds as examples, a recent study demonstrated that withaferin A, the main bioactive component isolated from the ayurvedic medicine Withania Somnifera, has a potent hepatoprotective effect in both MCD diet-induced NASH and HFD-induced NASH, while other studies revealed that glycyrhrizin or its active metabolite glycyrrhetinic acid were both shown to have a potent hepatoprotective effect in both MCD diet-induced NASH and in HFD-induced NAFLD models. In addition, baicalein attenuated MCD diet-induced NASH in rats via enhancing NRF2 signaling and protected AML-12 cells from lipotoxicity by suppressing ER stress and Thioredoxin interacting protein/NLRP3 inflammasome activation. The multiple component TCM is exemplified by Picrocroha kurroa, which is used in Indian Ayurvedic medicine for the treatment of digestive problems, was recently demonstrated to potentially lower HFD-induced hepatic lipid accumulation. Shenlin Baizhu powder, a widely used classical TCM formulation, suppressed p38 mitogen-activated protein kinase signaling in NASH and TJ-9 (termed as “Xiao Chai Hu Tang” in China and
“Sho-saiko-to” in Japan), which is in clinical studies for treating hepatitis C, also showed effects in experimental NAFLD and NASH models. In addition, tanshinone IIA demonstrated protective effects in improving NAFLD in preclinical models. Similarly, Bofutsushosan (kampo medicine), Jiang Tang Xiao Ke Granule, Chaihu-Shugan-San Decotion, Chinese bayberry juice, wolfberry, shirazi’s thyme, ginger, and ginseng or ginseng-derived active component ginsenoside Rb2 and Rg1, have shown potential for improving obesity-related metabolic syndrome and NAFLD that deserves further study. Furthermore, a meta-analysis of 8 RCTs with a total of 800 patients suggested that danshen, a TCM for improving obesity-related metabolic syndrome and NAFLD, is effective. Similarly, Ke Granule, Chaihu-Shugan-San Decotion, Chinese bayberry juice, wolfberry, shirazi’s thyme, ginger, and ginseng or ginseng-derived active component ginsenoside Rb2 and Rg1, have shown potential for improving obesity-related metabolic syndrome and NAFLD that deserves future multicenter large-sample RCTs.

3. How to develop THM for treating NAFLD?

3.1. Reverse-pharmacology-guided approach

In contrast to the “bench-to-bedside” process used for modern drug development, most THM already in the clinic, lack preclinical evidence and large-scale randomized clinical trials to further verify their safety and efficacy. The advantages of a reverse pharmacology approach for clinically-used THM features a “beside (clinical efficacy)-to-bench (preclinical phenotype and mechanism)” approach. In the case of THM for treating NAFLD, most THM, although claimed to be hepatoprotective in treating liver diseases in traditional practice, the mechanisms have not been comprehensively defined. Due to a general lack modern research evidence, most market-available hepatoprotective THM are prescribed or used over-the-counter under general directions for treating acute or chronic liver diseases without any clearly-defined types of liver diseases. Although liver diseases share some common pathological mechanisms, different types of liver diseases vary in their pathological processes and thus the drug-gable therapy targets differ. To determine the molecular mechanisms and which types of liver disease are suitable for being treated by each THM, more studies are urgently needed using preclinical NAFLD models to guide the clinical use of THM, a process of reverse pharmacology. In addition, the reverse pharmacokinetics analysis of THM also benefits the clinical use, target/mechanism study and THM-derived drug discovery as reviewed previously.

3.2. Experiment design for testing the role of THM in treating NAFLD

NAFLD is one type of common chronic liver disease. To better support the hepatoprotective effects and explore the mechanisms of THM (which is already used in clinics), preclinical studies based on the reverse-pharmacology-guided approach are needed. The experiment designs for testing the role of THM in treating NAFLD is discussed in detail below.

3.2.1. NAFLD model choice

In the clinic, the large majority of NAFLD patients are obese, many NAFLD patients are lean. To better mimic pathological characters of the predominant obese NAFLD patients, most preclinical NAFLD animal models have obesity and obesity-associated metabolic syndrome such as insulin resistance and glucose intolerance. There are also NAFLD models that do not show increased body weight and/or insulin resistance. For example, NAFLD mice maintained on a MCD diet have decreased body weight, and are free of insulin resistance. The most frequently-used NAFLD models and their respective characteristics (which aspect could be emphasized by each model) were reviewed previously.

3.2.2. Drug dosing design

THM choice, drug dose, drug dosing route, and drug dosing regimen are the major concerns for THM drug delivery design. A THM for anti-NAFLD effects could be mainly from known hepatoprotective herbs, repurposing from traditional herbs with unknown hepatoprotective effects or herb-derived active components or metabolites. Drug doses should be designed based on publications related to the investigated THM. Short-term pilot studies for testing the effective dose or dose-dependency of THM in treating NAFLD are preferred for starting the full investigation of THM in treating NAFLD, especially by using the MCD diet-induced NASH model. A two-week MCD diet feeding could be long enough to cause significant serum ALT and AST increases for testing the efficacy of the pilot dosing method.

NAFLD animal models are usually long-term, and thus daily intravenous injection is difficult to carry out in rodent models. Oral intake by gavage, dietary supplementation as well as intraperitoneal injection are the most-frequently-used dosing methods for THM in mouse models. Dose regimen choice could be divided into two major types based on whether the THM is tested for examining the preventive effect or therapeutic effect. For evaluation of a preventive effect, mice are treated with a THM coincident with the induced onset of the disease or prior to the induced onset of the disease until termination of the experiments. To examine the therapeutic effect of a THM, the disease is induced and then the THM is administrated. For example with the MCD diet-induced NASH model, THM is usually dosed from the first day of MCD diet feeding to test the preventive effect, while for therapeutic effect, THM needs to be administered after NASH is established by MCD diet feeding for 6 weeks. Body weight-independent effects of THM in treating NAFLD is also of concern, mainly for determining whether the hepatoprotective effect of a THM is the result or cause of body weight change. For the HFD-induced obese NAFLD model, the drug is usually dosed for a relatively-short time at the early stages of onset of disease symptoms prior to body weight change, and any early anti-NAFLD markers such as ALT, AST, hepatic triglyceride or total cholesterol are measured. It is notable that the MCD diet-induced NASH model is a lean NASH model. This model could be relatively feasible for examining whether a THM-reduced MCD diet-induced NASH is due to body weight change.

3.2.3. New technology and omics-based high throughput screening for target/mechanism exploration

Evolution of new technologies have yielded multiple types of omics analysis (metagenomics, transcriptomics, metabolomics, and proteomics) that facilitates the high throughput screening for target/molecular mechanism exploration. First, 16S ribosomal RNA analysis enables metagenomics analysis of gut microbiota. Exploration of the microbiome in both mouse and human NAFLD/NASH not only largely clarifies the roles of intestinal microbiota in metabolic diseases, but also provides...
pharmacological implications for herbs in treating NAFLD\textsuperscript{150}. Detailed microbiota changes have been clarified for many herbs such as resveratrol, berberine and curcumin, as described in Section 2.3.2 of this review. Second, RNA sequencing (RNA-Seq) using next-generation sequencing helps analysis of the cellular transcriptome, while single-cell RNA-Seq further deconstructs the transcriptomes of complex tissues at the single-cell level and thus could tell how different types of individual liver cells (hepatocytes, Kupfer cells, HSCs, monocytes) and even zonal differences in liver hepatocytes, contribute to NAFLD/NASH progression or treatment. Most recently, by using single-cell secretome gene analysis in combination with quantitative proteomics and liver RNA-Seq analysis, a marked presence of macrophages that were characterized with high levels of triggering receptor expressed on myeloid cells 2 was uncovered as a hallmark of mouse and human NASH\textsuperscript{191}. Third, metabolomic analysis of serum metabolomes of NAFLD patients and NASH mice identified two major subtypes of NAFLD and found markers that differentiate steatosis from NASH in each subtype\textsuperscript{192}. In addition, targeted lipidomic analysis is also a powerful way to determine how THM modulates lipid metabolism during treating NAFLD. For example, with \textit{Eclipta prostrata} as chemical probes in combination with lipidomic, it demonstrated the therapeutic potential of this THM in treating NAFLD and revealed four lipid species as potential biomarkers for NAFLD prognosis\textsuperscript{193}.

3.2.4. Hypothesis-based molecular mechanism exploration

Hypothesis-based target exploration is aimed to validate whether a known pharmacotherapy target is targeted by THM. The pathogenesis and pharmacotherapy targets of NAFLD were reviewed previously\textsuperscript{35} and summarized in Fig. 1. The NAFLD pathogenesis and molecular targets combined with the molecular or chemical information of THM can yield a hypothesis and a potential anti-NAFLD target, and then whether the hypothesis fits \textit{in vitro} or \textit{in vivo} can be examined. Hypothesis-based target exploration belongs to the reverse-pharmacology-guided approach. The hypothesis-based target exploration methodology was extensively used to investigate many herbs and the frequently hypothesized mechanisms are listed in Table 2 and Fig. 3.

4. Challenges and hints

Clinical trials need to be launched in order to determine whether a THM is effective for treating NAFLD\textsuperscript{194}. In general, many factors such as drug dose variance, inter-individual patient variability and differences between rodents and patients make clinical trial design challenging. Compounds used as diet supplements also need to be subject to clinical trials\textsuperscript{35}. In addition, most THM has a very low bioavailability, which possibly compromises their therapeutic effects. Notably, although some herbs, such as berberine and resveratrol, have low bioavailability (less than 1%), leading to lack of hepatic accumulation and concentrations far below the required effective dose, they still show hepatoprotective effects\textsuperscript{84,112}. This interesting phenotype drives studies to explore whether and how THM work without being systematically absorbed. It’s known that some THM directly work at least partially through gut microbiota or intestinal-liver signaling\textsuperscript{84,112,195}. On the other hand, THM that have multiple components may show pharmacological effect \textit{via} the combined actions of several components through multiple molecular pathways.

Figure 3  Role and mechanisms of frequently-studied traditional herbs in treating NAFLD. Four frequently-studied THM (silymarin, curcumin, berberine, and resveratrol) are registered in ClinicalTrials.gov for clinically testing their safety and efficacy in treating NAFLD. Berberine and silymarin were subjected to a phase 4 clinical trial. These four representative traditional herbs show hepatoprotective effects in improving NAFLD/NASH in preclinical rodent mouse models \textit{via} various molecular pathways mainly including cell death modulation \textit{(via} inducing autophagy or inhibiting hepatocyte apoptosis\textit{)}, lipid metabolism modulation \textit{(via} activating FXR, PPAR\textsubscript{A}, PPAR\textsubscript{G}, AMPK, SIRT1 or antagonizing LXR\textit{)}, anti-inflammation \textit{(via} inhibiting TLR/MYD88 or NLRP3 inflammasome pathway\textit{)}, anti-oxidative stress \textit{(via} activating NRF2\textit{)}, modulating liver-gut axis \textit{(via} changing microbiota composition, repairing leaky gut to reduce the release of LPS or harmful bacteria, modulating intestinal FXR signaling or SIRT1 signaling, or producing microbiota products such as active bile acids or herb drug metabolites\textit{).}
targets/mechanisms make THM study even more complicated\cite{196,197}, which is also an important point for THM study and clinical use, but not emphasized in detail in this review.

5. Summary

THM are valuable sources for developing novel anti-NASH drugs. Beyond the related and diverse philosophy systems to emphasize the holistic health balance, THM itself at least provides valuable sources of anti-NASH drugs, lead compounds or adjuvant components for novel drug discovery. Market-available THM are usually directed or prescribed for treating virus-induced hepatitis or generally-defined acute and chronic liver diseases. NAFLD is one chronic liver disease that includes many pathological aspects, including steatosis, lipotoxicity, oxidative stress, ER stress, and insulin resistance. Increasingly, herbal products have been studied via well-designed controlled trials that provide evidence supporting beneficial effects in liver disease. Due to the variance of complicated clinical trial conditions for drug dosing methods and patient choice, most results are still inconclusive. Thus, large-scale multicenter RCTs for THM are urgently needed, with two herbs (silymarin and berberine) already in phase 4 clinical trials. On the other hand, with increasing use of THM, THM-induced toxicity has gathered increasing attention that attracts both consumer attention and research attention. Hopefully with more and more extensive and well-designed reverse-pharmacology-guided research for THM, people will be able to use these ancient herbs in a more safe and rational way based on solid evidence.

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Author contributions

Tingting Yan conceived the manuscript and figures; Tingting Yan and Nana Yan wrote the manuscript; Ping Wang made the figures; Haiping Hao, Yangliu Xia and Guangji Wang reviewed and edited the manuscript; Frank J. Gonzalez supervised and edited the manuscript; Nana Yan wrote the manuscript; Ping Wang made the figures; Tingting Yan conceived the manuscript and figures; Tingting Yan.

Conflicts of interest

The authors declare no conflict of interest for publishing this manuscript.

Appendix A. Supporting information

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