Molecular Pharmacology

Title page

Gossypetin prevents the progression of nonalcoholic steatohepatitis by regulating oxidative stress and AMP-activated protein kinase.

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Running title page

Running Title: Gossypetin alleviates NASH as dual targeting agents.

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Abbreviation

ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic) acid; ACC, Acetyl-CoA carboxylase; Acyl, acyl-CoA dehydrogenase; Acox1, acyl-CoA oxidase 1; ADaM, allosteric drug and metabolite; ALT, Alanine aminotransferase; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; AST, Aspartate aminotransferase; AUC, Area under the curve; CNBr, Cyanogen bromide; CDHFD, Choline-deficient high fat diet; Col1a/3a, Collagen type I/III alpha 1; DHE, dihydroethidium; Cpt1, carnitine palmitoyltransferase 1A; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Drp1, Dynamin related
protein 1; EPSP, excitatory postsynaptic potential; Fis1, Mitochondrial fission 1 protein; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; H&E, Hematoxylin and eosin; JC1, 1,1',3,3'-Tetraethyl-5,5',6,6'-tetrachloroimidacarbocyanine iodide; LKB1, liver kinase B; LM, liver microsomes; MCD, methionine-choline deficient diet; MCP1, Monocyte chemoattractant protein 1; NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; NRF2, Nuclear factor erythroid 2-related factor 2; OCR, Oxygen consumption rate; OxLDL, oxidized low-density lipoprotein; PA, Palmitic acid; ROS, reactive oxygen species; SOD1, Superoxide dismutase; SREBP1c, Sterol regulatory element-binding protein 1; TMRE, Tetramethylrhodamine; TRX, thioredoxin; TXNIP, thioredoxin-interacting protein.
Abstract

Nonalcoholic steatohepatitis (NASH) is severe liver metabolic disorder, however, there are still no effective and safe drugs for its treatment. Previous clinical trials used various therapeutic approaches to target individual pathological mechanisms, but these approaches were unsuccessful because of the complex pathological causes of NASH. Combinatory therapy in which two or more drugs are administered simultaneously to patients with NASH, however, it carries the risk of side effects associated with each individual drug. To solve this problem, we identified gossypetin as an effective dual-targeting agent that activates AMP-activated protein kinase (AMPK) and decreases oxidative stress. Administration of gossypetin decreased hepatic steatosis, lobular inflammation and liver fibrosis in the liver tissue of mice with CDHFD and MCD diet-induced NASH. Gossypetin functioned directly as anti-oxidant agents, decreasing hydrogen peroxide and palmitate-induced oxidative stress in the AML12 cells and liver tissue of MCD diet fed mice without regulating the antioxidant response factors. In addition, gossypetin acted as a novel AMPK activator by binding to the allosteric drug and metabolite (ADaM) site, which stabilizes the activated structure of AMPK. Our findings demonstrate that gossypetin has the potential to serve as a novel therapeutic agent for NAFLD/NASH.

Significance statement

This study demonstrates that gossypetin has preventive effect to progression of NASH as a novel AMPK activator and antioxidants. Our findings implicate that simultaneous activation of AMPK and oxidative stress using gossypetin has the potential to serve as a novel therapeutic approach for NAFLD/NASH patients.
1. Introduction

Excessive caloric intake contributes to the dysfunction of metabolism in the body, resulting in various metabolic disorders, such as obesity, diabetes, and nonalcoholic fatty liver disorder (NAFLD)(Paschos and Paletas, 2009). NAFLD is highly related to insulin resistance of liver and its symptoms can be reversed through medication and lifestyle changes(Brunt, 2010; Utzschneider and Kahn, 2006). However, if its symptoms are aggravated, then NAFLD can progress to nonalcoholic steatohepatitis (NASH)(Wree et al., 2013). NASH exhibits more severe symptoms related to hepatic steatosis as well as chronic inflammation and fibrosis in the liver, leading to liver cirrhosis and hepatocarcinoma(Peng et al., 2020; Schuster et al., 2018). Those multiple pathologies are the result of various causes such as lipid metabolic dysfunction, insulin resistance, oxidative stress, and inflammation(Fraile et al., 2021). Consequently, the search for a key regulator of NASH progression and the identification of the optimal timing for therapy present significant challenges. For this reason, it is important to develop the preventive and therapeutic agents for NASH, however, there are currently no available drugs approved by the United States Food and Drug Administration (FDA) specifically for the treatment of NASH (Younossi et al., 2018).

Flavonoids, which are metabolites derived from natural products, have the potential to be applied as preventive and therapeutic agents for metabolic disorders such as obesity, diabetes, and NAFLD/NASH(Mahboob et al., 2023; Sandoval et al., 2020). Pre-clinical studies and clinical trials have reported that dietary flavonoids can improve insulin sensitivity, dysregulated lipid metabolism, and mitigate oxidative stress in the pathological conditions associated with metabolic disorders(Mahboob et al., 2023). Moreover, flavonoids offer the advantage of long-term safety, making them a suitable candidate for preventive metabolic disorder supplementation over an extended duration(Ross and Kasum, 2002).
Gossypetin is one of flavonoid derivatives extracted from the flower of hibiscus (Hibiscus sabdariffa) (Gómez-Aldapa et al., 2018). It has been reported that gossypetin possesses anti-bacterial effect (Gómez-Aldapa et al., 2018), anti-atherosclerotic effect (Lin et al., 2021), inhibitory effect of neuraminidase from influenza virus (Jeong et al., 2009) and protective effect of DNA damage (Khan et al., 2013). However, the biological activity of gossypetin and its underlying mechanism on metabolic disorder is still unclear. In this study, we identified gossypetin, which is a flavonoid metabolite from plant material, as an effective dual-targeting agent that could prevent NASH pathology.

2. Materials and methods

2.1. Animals

Four-week-old male C57BL/6 mice were purchased from Central Lab Animal Inc. (Seoul, Korea). After 2 weeks of adaption, mice in the control group were fed a standard chow diet and the MCD diet (Research Diets, New Brunswick, NJ, United States) for 4 weeks or CDHFD diet (Research Diets) for 3 months to induce NASH. Gossypetin was orally administered at a dose of 20 mg/kg/day for 4 weeks with the MCD diet and for 3 months with the CDHFD diet to evaluate its preventive effect. After drug treatment, blood samples were collected by retrobulbar bleeding, and serum was collected after centrifugation (3000 rpm, 20 min). ALT and AST levels in serum samples were measured by SCL Health Inc. (Seoul, Korea) to examine lipotoxicity. All animal experiments were conducted according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy and the principles of the NIH Guide for the Care and Use of Laboratory Animals. All mice were maintained under conventional conditions at the POSTECH animal facility under institutional guidelines (POSTECH IACUC protocol code 2022-0073). For the pharmacokinetic and tissue distribution study, C57BL/6 mice (7-week-old, male) were purchased from Samtako Co. (Osan, Kyunggi-do, Korea). Animals were acclimatized for 1
week in an animal facility at Kyungpook National University. Food and water were available ad libitum. All procedures were approved by the Animal Care and Use Committee of Kyungpook National University (KNU IACUC protocol code 2022-0179).

2.2. Drug treatment

Gossypetin was synthesized by Boc sciences (Shirley, NY, USA) and dissolved in DMSO (Sigma-Aldrich) for cell-based experiments and mouse administration. To investigate its effects, gossypetin (20 mg/kg) or vehicle (1% carboxymethylcellulose) was orally administered to mice in four different groups (normal diet feeding group, normal diet + gossypetin, MCD diet feeding group, MCD diet + gossypetin) for 4 weeks (n=17-20).

2.3. Histology

Mouse liver tissue was fixed in 4% paraformaldehyde (PFA) after sacrifice and embedded in paraffin for H&E and Sirius red staining. Paraffin blocks were sectioned (4 μm thick) and stained with H&E to measure the NAS. NAS was measured following the criteria of De and Duseja (De and Duseja, 2019) using three variables: hepatocellular steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning (1-2). NAS ≥ 5 is correlated with a diagnosis of NASH, whereas NAS ≤ 3 is correlated with a diagnosis of non-NASH. To measure the level of fibrosis pathology, paraffin sections were also stained with Picosirius red solution (IHC WORLD, Ellicott City, MD, USA). Sections were deparaffinized with xylene and dehydrated with decreasing concentrations of ethanol (100% to 70%). Dehydrated sections were stained with Weigert’s iron hematoxylin solution (IHC WORLD) and washed with running tap water before Sirius red staining. Sections were then washed with 0.5% acetic acid solution before being dehydrated with 100% ethanol. The sections were mounted onto slide glass with Optic mount S3 (BBC Biochemical, Mount Vernon, WA, USA) for further quantitative imaging analysis. All images were taken under an Axioplan2 microscope (Zeiss Axio Scan Z.1, Jena, Germany). Liver tissues were embedded and frozen in optimal cutting temperature (OCT)
compound (Sakura Finetek USA, St, Torrance, CA, USA) on dry ice. OCT blocks were sectioned (10 μm thick) and stained with oil red O (Sigma-Aldrich) to measure liver steatosis. Frozen sections were air-dried at room temperature and then fixed in ice-cold 10% formalin (Sigma-Aldrich). Fixed sections were rinsed immediately three times with distilled water. Then, sections were placed in 100% propylene glycol (Sigma-Aldrich) and stained in prewarmed oil red O solution in a 60°C oven. Sections were then differentiated in 85% propylene glycol solution and then rinsed twice with distilled water. Next, sections were stained with Mayer’s modified hematoxylin solution (Abcam, Cambridge, UK) and washed under running tap water. The sections were mounted onto slide glass with Dako (Agilent, Glostrup, Denmark) for further quantitative imaging analysis.

For other liver tissues, after they were fixed in 4% PFA, they were placed into 30% sucrose in PBS at 4°C until they settled. Tissues were frozen in OCT compound and stored at −80°C for storage. Liver tissue (10 μm thick) was cut and prepared on slide glass. Tissue sections were then permeabilized and blocked using a blocking solution (5% fetal bovine serum, 3% bovine serum albumin, and 0.3% Triton X-100 in PBS) for 1 h at room temperature. Primary antibody incubation was performed overnight at 4°C using anti-F4/80 antibody (Biolegend, San Diego, CA, USA). After washes, sections were incubated with secondary antibody (Invitrogen, Waltham, MA, USA) for 1 h at room temperature in the dark. Slides were counterstained in Hoechst and then mounted in fluorescence mounting medium (Dako). All images were taken using an Axioplan2 microscope. Fluorescent intensity was analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.4. Hydroxyproline assay

To examine whether gossypetin decreases fibrosis pathology in the liver tissue of MCD diet-fed mice, hydroxyproline content was measured. Total hepatic collagen content was determined to measure the relative levels of hydroxyproline, a precursor of collagen. Liver samples (50 mg)
were homogenized in distilled water (100 μl) and hydrolyzed in 10 N HCl at 120°C for 3 h. The hydroxyproline content was determined as described in a previous report (Brown et al., 2001).

2.5. Free fatty acids quantification

Free fatty acids level in the liver tissue were measured through free fatty acid quantification kit (Sigma-Aldrich), according to the manufacturer’s instructions.

2.6. Oxygen consumption rate (OCR measurement)

AML12 cells were seeded on Seahorse XF 96-well plate overnight. Then, the cells were cotreated together with BSA control or 250μM palmitate and gossypetin for 24 hours. Before analysis, cells were washed and equilibrated for 1 h at 37°C with XF base medium (102353-100; Agilent technologies) supplemented with and 25mM glucose (Sigma-Aldrich), 1X GlutaMAX (Gibco), 1mM sodium pyruvate (Sigma-Aldrich) (pH 7.4). Then, the pre-hydrated sensor cartridge loaded with the mitochondrial inhibitors to deliver a final concentration of 1μM oligomycin (Sigma-Aldrich), 1μM FCCP (Sigma-Aldrich), and 0.5μM rotenone (Sigma Aldrich) + 0.5μM Antimycin A (Sigma-Aldrich) was placed on the XF 96-well plate and OCR was measured before and after the sequential injection of mitochondrial inhibitors. OCR measurements were normalized with the total cell number and analyzed using Wave 2.6.0 software, Agilent technologies.

2.7. JC-1 staining and TMRE staining

To evaluate the mitochondrial function following the treatment of gossypetin, 1,1',3,3'-Tetraethyl-5,5’,6,6’-tetrachloroimidacarbocyanine iodide (JC-1) staining experiment and TMRE staining were performed using mitochondrial staining kit (Sigma-Aldrich). AML12 cells were seeded on the chip and the mitochondrial membrane dysfunction was induced by the treatment with palmitate (250 μM). JC-1 dye or TMRE dye was treated to AML12 cells on chip after the treatment of gossypetin along with previously-treated palmitate for 24hr. Through the staining,
JC-1 aggregates are detected with the red fluorescence signals while JC-1 monomers are detected with the green fluorescence signals. The ratio of red/green fluorescence in the mitochondria is considered as the level of mitochondria membrane potential. The ratio of red/green fluorescence was measured through ImageJ program.

2.8. Cell culture

AML12 mouse hepatocytes were cultured with Dulbecco’s Modified Eagle Medium/F12 (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone), 10% penicillin/streptomycin (Welgene, Gyeongsan-si, Korea), 20 mg of dexamethasone (Sigma-Aldrich), and 5 ml of insulin-transferrin-selenium in a 5% CO₂ incubator with a humidified atmosphere at 37°C.

2.9. Evaluation of antioxidant effects in vitro and in vivo

To evaluate the antioxidant effects of gossypetin, the DPPH and ABTS assays were conducted. First, 0.2 M DPPH) was prepared in methanol and mixed with 0, 2.5, 5, 10, 12.5, or 25 μM gossypetin and ascorbic acid (Sigma-Aldrich) in a 1:1 ratio. The mixtures were incubated for 30 min in the dark. After incubation, 200 μl of each DPPH mixture were loaded into 96-well plates, and the optical density was measured at 517 nm with TECAN Infinite M200 PRO (TECAN, Männedorf, Switzerland). The ABTS assay was conducted using an ABTS antioxidant assay kit (Zen-Bio Inc., Durham, NC, USA) according to the manufacturer’s protocol. To measure oxidative stress at the cellular and tissue levels, we conducted a CellRox experiment and DHE staining experiment. Oxidative stress was induced in AML12 cells by treatment with palmitate (Sigma-Aldrich) and hydrogen peroxide (Sigma-Aldrich). Cells were pretreated with gossypetin for 24 h before oxidative stress was induced. After medium replacement, AML12 cells were incubated with CellRox reagent. Induced oxidative stress was measured by detecting the fluorescence intensity using an Axioplan2 microscope. DHE was used to detect oxidative stress.
in liver tissue. Liver tissue sections were prepared and stained with diluted (10 μM) DHE for 1 h. The fluorescence intensity was analyzed using ImageJ.

2.10. Molecular docking

Crystal structures of human AMPK (code: 5iso) were prepared from the RCSB Protein Data Bank. Molecular docking and calculation of the predicted binding energy were performed using AutoDock Vina in PyRx software to identify the docking position in AMPK. The grid map for the docking was covered on the ADaM site in the N-terminus of AMPK. The docking results were visualized using the PyMol visualization system. Hydrogen bonds were predicted by PyMol software.

2.11. Western blotting

Cells were lysed with lysis buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 5 mM EDTA, and a protease inhibitor tablet, followed by sonication. The protein concentration of the lysate was determined using Bradford reagent (AMERSCO, Framingham, MA, USA). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Pall Corporation, New York, NY, USA), and incubated with blocking buffer (5% nonfat dry milk in TBS and 0.1% Tween 20) for 30 min. Western blotting was performed using primary antibodies against AMPK, phospho-AMPK, LKB, phospho-LKB (all from Cell Signaling Technology, Danvers, MA, USA), and GAPDH (Bethyl Laboratories, Montgomery, TX, USA). Secondary rabbit (Promega, Madison, WI, USA), rat, and goat antibodies (both from Bethyl Laboratories) were used, and detection was performed using SUPEX ECL reagent (Neuronex, Goryeong, Korea) and an ImageQuant LAS-4000 (GE Healthcare, Chicago, MA, USA), according to the manufacturer’s instructions. The integrated blot density was quantified by ImageJ.

2.12. Reverse transcription (RT-PCR) and real-time quantitative PCR (qPCR)
Total RNA was isolated using NucleoZOL reagent (Takara Bio Inc., Kusatsu, Shiga, Japan). RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer’s instructions. For detection and quantification, a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). Real-time qPCR data were analyzed using the comparative C<sub>T</sub> method.

2.13 Measurement of 26S proteasome activity

To measure the 26S proteasome activity under the treatment with gossypetin and A-769662, AML12 were seeded on 12 well plate and were treated with DMSO or drugs. Proteasome-Glo Cell-Based Assay were performed following the manufacturer’s instructions to measure the chymotrypsin-like activity of the 26S proteasome using N-Suc-LLVY-aminoluciferin as the substrate (Promega, Madison, WI).

2.14. Extracellular field potential recordings

Using 3 weeks-old male mice, transverse hippocampal slices (400 μm) were prepared for extracellular field potential recordings. After decapitation, brains were rapidly removed and sliced by vibratome using ice-cold low-Ca<sup>2+</sup>/high-Mg<sup>2+</sup> dissection buffer (5 mM KCl, 1.23 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 10 mM dextrose, 0.5 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, and 212.7 sucrose). Slices were transferred to a holding chamber in an incubator containing oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) artificial CSF (ACSF; 124 mM NaCl, 5 mM KCl, 1.23 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, and 10 mM dextrose) at 28–30°C for at least 1 h before recording. Slices were transferred to a recording chamber perfused continuously with oxygenated ACSF (28-30 °C) at a flow rate of 1.5 ml/min. The fEPSP were evoked by Schaffer collateral (SC) stimulation (0.2 msec pulses) using a concentric bipolar electrode. Synaptic potential of CA1 was recorded with ACSF-filled microelectrodes, an AM-1800 microelectrode amplifier (A-M Systems, Sequim, WA, USA), a PG 4000A stimulator (Cygnus Technology, Southport, NC, USA), and a SIU-90 isolated current source (Cygnus Technology). Synaptic
responses were collected at 0.07 Hz with a stimulation intensity that yielded a 50% maximal response. Paired pulse ratio was measured after bath application of AICAR or gossypetin for 30 minutes for stable responses. IGOR software (Wavemetrics, Portland, OR, USA) was used for analyzing the responses.

2.15. Pharmacokinetics and tissue distribution of gossypetin

To investigate the pharmacokinetics and the tissue distribution of gossypetin, forty-two male C57BL/6 mice (20-24g) were fasted with water ad libitum for 16 h before the oral administration of gossypetin and were randomly divided into seven groups (n = 6 per each sampling time point). Mice were administered with a gossypetin suspension in 1% carboxymethylcellulose at a dose of 20 mg/kg via oral gavage and blood samples (approximately 0.1 mL) were collected at 0, 0.25, 0.5, 1, 2, 4, 8, 24 h via the abdominal artery. Subsequently, liver, brain, kidney, heart, muscle, and adipose tissues were isolated, weighed, and homogenized with two volume of ice-cold saline containing 5 mg/mL ascorbic acid using a tissue grinder. Blood samples were centrifuged at 12,000 g for 1 min to separate plasma. Aliquots of plasma (30 µL each) and liver, brain, kidney, heart, muscle, adipose tissue homogenate samples (100 µL each) were added to 30 µL of internal standard solution (IS; quercetin 100 ng/mL in acetonitrile) and were vortexed for 1 min. The mixture was added to 1 mL of ethyl acetate and vigorously mixed for 5 min. After centrifugation at 16,000 g for 5 min, a 950 µL aliquot of the supernatant was dried using Speed vac (20 mbar, 60 °C) for 25 min. The residue was reconstituted with 100 µL of 80% acetonitrile containing 5 mg/mL of ascorbic acid for 5 min and centrifuged at 16,000 g for 5 min. Aliquots (5 µL) of the supernatant was injected into an Agilent 6470 triple quadrupole liquid chromatography-mass spectrometry (LC–MS/MS) system (Agilent, Wilmington, DE, USA). Gossypetin and quercetin (IS) were separated on a Synergi Polar RP column (2.0 × 150 mm, 4 µm particle size; Phenomenex, Torrence, CA, USA) using a gradient elution of distilled water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 0.3 mL/min. Gradient elution was as follows:
Quantification of the analyte peaks was carried out at m/z 317.1 → 166.9 for gossypetin and m/z 301.1 → 151.0 for quercetin (IS) in a negative ionization mode with optimized fragmentor of 135 V and collision energy of 20 eV, respectively. The standard calibration curves for gossypetin were prepared in the range from 3 nM to 1500 nM using the blank plasma, liver, brain, kidney, heart, muscle, and adipose tissue homogenates, respectively, and showed good linearity in all standard curves (r² > 0.992). The coefficient of variance for the interday precision and accuracy was below 15%. Concentrations of gossypetin in the plasma, liver, brain, kidney, heart, muscle, and adipose tissue homogenates samples were calculated from the linear regression equation of gossypetin standard curves for the respective plasma or tissue using the ratio of the peak areas of gossypetin and IS. The pharmacokinetic parameters of gossypetin in plasma and liver, brain, kidney, heart, muscle, adipose tissues were calculated by the non-compartmental analysis (WinNonlin® 5.1; Pharsight, Mountain View, CA, USA).

2.16. Stability of gossypetin

The stability of gossypetin in phosphate-buffered saline (PBS), mouse plasma, and mouse liver microsomes (LM) was measured. Gossypetin (100 μM) was added to 50 μL of PBS and mouse plasma, and the mixture was incubated for 0, 0.5, 1, 1.5, and 2 h. To investigate the metabolic stability of gossypetin, a reaction mixture was prepared by combining 90 μL of 50 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl₂, 10 μg/mL alamethicin, 1 μM gossypetin, and 0.5 mg/mL of mouse LM. The mixture was preincubated for 5 min and then the metabolic reaction was initiated by adding 10 μL of the NADPH generating system (1 mM NADPH, 5 mM glucose 6-phosphate, 1 mM NADP⁺, 1.0 kU glucose 6-phosphate dehydrogenase) and 10 mM UDPGA. The reaction was incubated at 37 °C for 0, 0.5, 1, 1.5, and 2 h. After incubation, the reaction mixture was quenched by adding 50 μL of ice-cold saline containing 5 mg/mL ascorbic acid and 50 μL of ice-cold acetonitrile. Subsequently, the mixture was added to 1 mL of ethyl acetate and vigorously mixed for 5 min. Following centrifugation at 16,000 g for 5 min,
a 950 μL aliquot of the supernatant was dried and reconstituted with 100 μL of 80% acetonitrile for 5 min. After centrifugation at 16,000 g for 5 min, 5 μL aliquots of the supernatant were injected into an LC-MS/MS system to measure the gossypetin concentration. After incubation, reaction mixture was quenched with 50 μL ice-cold saline containing 5 mg/mL ascorbic acid and 50 μL ice-cold acetonitrile and subsequently added to 1 mL of ethyl acetate and vigorously mixed for 5 min. After centrifugation at 16,000 g for 5 min, a 950 μL aliquot of the supernatant was dried and reconstituted with 100 μL of 80% acetonitrile for 5 min and centrifuged at 16,000 g for 5 min. Aliquots (5 μL) of the supernatant was injected into LC–MS/MS system to measure the gossypetin concentration. The elimination slope (K) was calculated from the log-transformed gossypetin amount versus time profile. The metabolic activity, expressed as intrinsic clearance (Clint), and hepatic extraction ratio were calculated according to the following reference (Huang et al., 2015).

2.17. Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean. Comparisons between two groups were analyzed by a two-tailed unpaired Student’s t-test. For comparisons between more than two groups, one-way or two-way analysis of variance was performed with Tukey’s test. p < 0.05 was considered statistically significant.

3. Results

3.1 Gossypetin attenuated NASH pathology in the choline deficient high fat diet and methionine-choline deficient diet-induced mice model.

NASH shows common pathologies, such as hepatotoxicity, liver steatosis, chronic inflammation, and fibrosis in the liver tissue. To evaluate its preventive effect of gossypetin on NASH, gossypetin was orally administrated to choline deficient high fat diet (CDHFD) and methionine-choline deficient (MCD) diet-fed mice. Before the proper concentration of gossypetin was selected, we performed the concentration dependent study of gossypetin. When we treated 5 mg/kg of gossypetin to CDHFD mice, there were no changes to the pathology of
NASH (Supplemental Fig. 1) and the anti-NASH effect started to show from 20 mg/kg (Supplemental Fig. 4). In addition, we confirmed the pathological changes after the treatment of gossypetin (5, 20, and 50 mg/kg) to MCD mice (Supplemental Fig. 2A). Similar to the results of the CDHFD model, the anti-NASH effect of gossypetin started to show in the MCD model at the concentration of 20 mg/kg and there were no dramatic differences when compared with 50 mg/kg treatment (Supplemental Fig. 2). Through these results, we determined that the appropriate concentration of gossypetin for animal treatment would be 20 mg/kg. While CDHFD and MCD diet feeding resulted in a reduction of body weight, the administration of gossypetin did not affect the body weight or the tissue weight (Fig. 1B-C and Supplemental Fig. 3) except for the spleen. Interestingly, the weight of the spleen per body weight was increased in the MCD mice, but the gossypetin treatment decreased the weight of spleen (Supplemental Fig. 3E). After the drug treatment, we investigated the level of liver toxicity markers (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) in serum to confirm the positive effect of gossypetin on liver injury. Serum AST and ALT levels were increased by CDHFD and MCD diet feeding, and these changes were alleviated by gossypetin administration (Fig. 1D and Supplemental Fig. 4D-E). We next performed hematoxylin and eosin (H&E) staining to measure the NAFLD activity score (NAS), which was calculated by evaluating three of NASH pathologies, namely steatosis, hepatocyte ballooning, and lobular inflammation. NAS of gossypetin-treated mice was lower than that of untreated mice (Fig. 1E-G and Supplemental Fig. 4F-G). Oil red O staining data revealed that lipid accumulation was remarkably decreased in gossypetin-treated mice group (Fig. 1H, I and Supplemental Fig. 2F, G). In parallel with the oil red O staining data, the level of free fatty acid was decreased in the liver tissue of gossypetin-treated mice (Fig. 1J). To clarify the inhibitory effect on lipid metabolism, we measured the gene expression related to lipogenesis (Srebp1c and Acyl) and β-oxidation (Cptα and Acox1) in the liver tissue. While the treatment of gossypetin decreased the gene expression of lipogenesis genes, the genes related to β-oxidation was increased (Fig. 1K). These results indicate that both short-term and long-term treatments of gossypetin could prevent the
progression of NASH pathology.

3.2 Gossypetin decreased MCD diet-induced chronic inflammation and fibrosis in the liver tissue.

H&E staining analysis of liver tissue revealed that lobular inflammation was decreased in mice treated with gossypetin. To confirm the effect of gossypetin on liver inflammation, we performed immunohistochemistry to explore infiltrated F4/80-positive macrophages and measured the expression of genes related to chronic inflammation in NASH pathological condition. Consistent with the results of H&E staining, the number of F4/80-positive infiltrated macrophages was decreased in the liver tissue of gossypetin-treated mice group (Fig. 2A-B). In addition, the size of colonies with densely packed macrophages in the liver tissue of MCD mice was decreased in the liver tissue of gossypetin treated mice. We also observed the expression of the chronic inflammation related genes, such as *Mcp-1* and *Cd68*. The expression of these genes was decreased by gossypetin treatment, illustrating that the gossypetin can also attenuate chronic inflammation in liver tissue (Fig. 2C). We also investigated whether gossypetin alleviates liver fibrosis in mice with NASH. In the liver tissue of MCD diet-fed mice, sirius red staining revealed an increase in the liver fibrosis near blood vessels. The staining area (%) was decreased in the liver tissue of gossypetin treated mice (Fig. 2D-E). To confirm the inhibitory effect of gossypetin on collagen synthesis, we measured the contents of hydroxyproline, a precursor of collagen, and the expression of the collagen 1α and collagen 3α genes. Gossypetin decreased hydroxyproline levels and collagen gene expression (Fig. 2F-G). These data demonstrated that gossypetin dramatically reduces the pathology of NASH in MCD diet-fed mice.

3.3 Gossypetin exhibited powerful antioxidant effects.

Excessive free fatty acid levels induce mitochondrial dysfunction leading to oxidative stress in the NASH pathological condition(Bhatti et al., 2017). Oxidative stress is well known to cause
hepatic injury as well as inflammation by increasing the production of pro-inflammatory cytokines (Chen et al., 2020). To assess the antioxidant effect of gossypetin, we conducted 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic) acid (ABTS), and CellRox assays to measure oxidative stress in vitro and in vivo. When we evaluated the antioxidant effect of gossypetin using the DPPH and ABTS assays, the results show that gossypetin had greater scavenging effect on ROS than the positive control (Fig. 3A-B). To investigate the effect of gossypetin on oxidative stress at the cellular level, we pretreated AML12 cells with gossypetin for 24 hours before exposure to hydrogen peroxide, an oxidative stress-inducing agent (Supplemental Fig. 5A and Fig. 3C). The level of oxidative stress was analyzed by measuring the fluorescence intensity following treatment with CellRox reagent. The fluorescent intensity was lower in gossypetin-treated cells than in DMSO-treated cells (Fig. 3C-D). When the oxidative stress is induced in cells, an antioxidant response mechanism is activated by the NRF2. Under normal conditions, NRF2 constitutively undergoes proteasomal degradation via Keap1-mediated repression. However, excessive ROS production inhibits the proteasomal degradation of NRF2 and stabilizes the NRF2 levels. Nuclear-translocated NRF2 acts as a transcriptional factor for antioxidant response-related genes such as superoxide dismutase 1 (SOD1) (Bellezza et al., 2018). Thus, we confirmed whether gossypetin could regulate factors related to antioxidant responses dependent on NRF2 signaling. The results illustrated that gossypetin did not affect the levels of NRF2 or its target protein SOD1 (Supplemental Fig. 5B-C). These data indicate that gossypetin directly reduces oxidative stress in AML12 cells rather than activating NRF2 dependent antioxidant responses. To ascertain the antioxidant effect of gossypetin under a condition similar to the actual pathological condition of NASH, we induced oxidative stress in AML12 cells via palmitate treatment. Pretreatment with gossypetin decreased palmitate-induced oxidative stress (Fig. 3E, F). We also evaluated the antioxidant effect of gossypetin in the liver tissue of MCD diet-fed mice by conducting dihydroethidium (DHE) staining. Consistent with our previous results during cellular experiments, lipid peroxidation was decreased in the livers of gossypetin-treated MCD diet-fed
mice (Fig. 3G-H).

3.4. Gossypetin improves dysregulated mitochondrial function induced by palmitate treatment.

It has been reported that palmitate induces oxidative stress by depolarization of the mitochondrial membrane potential and further causes hepatic cell death in the NASH pathological condition (Ly et al., 2017). To ascertain the protective effect of gossypetin on mitochondria function, we measured the mitochondrial respiration after the induction of oxidative stress using palmitate. When gossypetin was treated to AML12 cells that were pretreated with palmitate, oxygen consumption rate was rescued (Fig. 4A-B). We also performed to TMRE and JC-1 staining experiment to confirm whether gossypetin induces mitochondrial function. While the production of JC-1 aggregates (red fluorescence) was decreased, the amount of monomer (green fluorescence) was increased in the palmitate treated AML12 cells, suggesting that palmitate reduced mitochondrial membrane potential. Comparing with palmitate-treated cells, treatment of gossypetin improved mitochondrial membrane potential (Supplemental Fig. 6). TMRE staining result also showed that dysregulated mitochondrial membrane potential was increased following treatment of gossypetin (Fig. 4C-D). Next, we examined the expression of genes related to mitochondrial biogenesis (Drp-1 and Fis1) using the liver tissue treated with gossypetin or vehicle. The gene expression of Drp-1 and Fis1 was decreased in the liver tissue of MCD diet-induced mice model, whereas, the relative fold changes were rescued in the liver tissue of gossypetin-treated mice to similar level with control mice (Supplemental Fig. 6C). Moreover, we next confirmed whether gossypetin reduced oxidative stress-induced hepatic cell death by measuring cleaved caspase-3. Cleaved caspase-3 levels were increased in the liver of MCD diet-fed mice, however, gossypetin treatment markedly decreased the number of cleaved caspase-3 positive cells (Fig. 4E-F). Taken together, these results implicate that the antioxidant effect of gossypetin protects hepatocytes from mitochondrial dysfunction and oxidative stress-induced hepatic cell death.
3.5. Gossypetin attenuated the dysfunction of lipid metabolism through AMPK activation.

AMP-activated protein kinase (AMPK) signaling pathway mainly regulates lipid metabolism during the progression of NASH (Herzig and Shaw, 2018). It has been reported that liver-specific AMPK activation dramatically reduces liver steatosis, chronic inflammation, and liver fibrosis (Garcia et al., 2019). This led us to examine whether AMPK activation is another pharmacological mechanism of gossypetin. To determine whether gossypetin induces AMPK phosphorylation, we treated gossypetin to AML12 with different concentrations of gossypetin. Gossypetin increased AMPK phosphorylation at a low concentration with an EC50 value of 0.43 \(\mu\text{M}\) (Fig. 5A-B). Consistently, AMPK phosphorylation was induced in the liver tissue of gossypetin treated mice (Fig. 5C-D). When we compared the gossypetin-induced AMPK phosphorylation to the known AMPK activators, AICAR and A-769662, the increase in the level of AMPK phosphorylation was higher in gossypetin-treated cells (Fig. 5E-F and Supplemental Fig. 7A-B). Treatment with gossypetin also showed greater improvements in NASH pathologies, like liver steatosis, fibrosis, and inflammation, than the treatment with AICAR or A-769662 at the same concentration (Supplemental Fig. 8). To elucidate the molecular mechanism of gossypetin, we established hypotheses on the regulation of AMPK phosphorylation. It is well known that AMPK phosphorylation is regulated by LKB1, which is an upstream kinase of AMPK. We confirmed whether gossypetin regulates the expression level or posttranslational modification of AMPK upstream kinases. Gossypetin treatment did not alter the levels of LKB and phospho-LKB (Supplemental Fig. 7C-D). Another possibility is direct regulation of the allosteric structure of AMPK by gossypetin as an ADaM site activator. It was previously reported that a small-molecule activator that binds to the ADaM site of AMPK stabilizes the activated AMPK structure (Gu et al., 2018). To investigate whether gossypetin can induce this stabilization, we predicted the binding affinity of gossypetin for the ADaM site of AMPK using the AutoDock program. The results illustrated that gossypetin could interact with
Ser108, Lys29, Lys31 and Asn48 of the ADaM site (Fig. 5G), and its binding energy was identical to that of the A-769662 and AICAR (Supplemental Fig. 7E-F). The CNBr bead binding assay was performed to ascertain the binding between AMPK and gossypetin. As presented in Fig. 5H, AMPK protein band was strongly detected in the gossypetin-conjugated CNBr bead sample, indicating gossypetin potentially binds directly to AMPK. These data indicate that gossypetin can activate AMPK by binding to its ADaM site and potentially ameliorate metabolic dysfunction by increasing AMPK phosphorylation.

To clarify the action mechanism of gossypetin depending on AMPK, we assessed the level of lipid accumulation through Oil Red O staining after treating gossypetin with compound C. When we inhibited AMPK activation using compound C, we observed an increase in lipid accumulation compared to the control cells. Notably, gossypetin treatment reduced lipid accumulation, but this effect was not observed in the cells where AMPK was inhibited (Fig. 6A-B). Consistent with the findings from Oil Red O staining, gossypetin treatment induced AMPK phosphorylation in AML12 cells. However, this effect was not observed in the cells where AMPK was inhibited (Fig. 6C-D). Additionally, we confirmed the phosphorylation level of Acetyl-CoA carboxylase (ACC) which is a AMPK target protein in the AMPK α1/2 double KO MEF after treatment with gossypetin. ACC phosphorylation is induced in the gossypetin-treated WT-MEF, however, ACC phosphorylation was disappeared in the AMPK α1/2 KO MEF (Fig. 6E-F). These results strongly indicate that gossypetin attenuates lipid accumulation in hepatocytes by relying on AMPK activation.

3.6. Gossypetin did not show off-target effects of well-known AMPK activators.

Although AICAR and A-769662 have been developed as AMPK activators, some reports show the off-target effects of these drugs independently from AMPK activation. Previously, it has been reported that A-769662 inhibits proteasome 26S activity independently of AMPK signaling pathway and induces off-target toxicity (Moreno et al., 2008). To confirm whether gossypetin has the same off-target effect, we confirmed the cell viability and 26S proteasome activity after
the treatment with gossypetin or A-769662. The treatment with high concentration of A-769662 decreased the cell viability while the treatment with same concentration of gossypetin did not show cytotoxic effect. To confirm the inhibitory effects of A-769662 and gossypetin on proteasome, chymotrypsin-like activity of the 26S proteasome was measured using N-Suc-LLVY-aminoluciferin as substrate. As shown in the previous study report, A-769662 reduced 26S proteasome activity, while the same concentration of gossypetin did not show the same effect (Supplemental Fig. 9). AICAR also has been reported to have an off-target effect on hippocampal synaptic transmission in rats (Gadalla et al., 2004). We examined whether gossypetin affects synaptic transmission like AICAR. By measuring field excitatory postsynaptic potential (fEPSP), we compared the effects of gossypetin and AICAR on synaptic transmission in the mouse hippocampus CA3-CA1 Schaffer-collateral synapse. It is noteworthy that AICAR reduced the paired-pulse ratio of fEPSP, indicating an increase in neurotransmitter release (Supplemental Fig. 10). However, gossypetin did not have the inhibitory effect on fEPSP at the same concentration. It is evident from these results that gossypetin did not exhibit off-target effects found in well-known AMPK activators and this implicates that gossypetin could be more selective and safer than AICAR or A-769662.

3.7. Pharmacokinetics and tissue distribution of gossypetin

Next, we measured the amount of gossypetin in the plasma and the liver in mice after the oral administration of gossypetin at a dose of 20 mg/kg to confirm the pharmacokinetics property. As shown in Fig. 7A, the concentration of gossypetin in the liver tissue was higher than the plasma gossypetin at early time but was eliminated faster than the plasma gossypetin, resulting in higher tissue to plasma area under concentration curves (AUC) ratio (T/P ratio, AUC$_{24\text{h, liver}}$/AUC$_{24\text{h, plasma}}$) of 1.73 and short elimination half-life (T$_{1/2}$) (Fig. 7C). In addition, the concentration of gossypetin in the liver tissue was higher than the EC$_{50}$ value of gossypetin (0.43 μM) for 1 h after its oral administration (20 mg/kg), suggesting the initiation of AMPK phosphorylation in the liver at 20 mg/kg dose of gossypetin. Since AMPK is also expressed in
the brain, kidney, heart, muscle, and adipose tissue (Steinberg and Carling, 2019), we also measured the distribution of gossypetin in the tissues that are known to express the AMPK. As shown in Fig. 7B, the gossypetin concentration-time profile in the brain, kidney, muscle, and adipose tissues are similar to that in plasma. Therefore, the maximum concentration (C_{max}) and AUC values of gossypetin in the brain, adipose tissue, muscle, heart, kidney were not different, but those in the heart was lower than that of plasma. Moreover, all the gossypetin concentrations in the plasma and tissues except for the liver was below the EC_{50} value of gossypetin (0.43 μM) (Fig. 7B-C). These results suggest that AMPK phosphorylation in the other tissues may not be initiated at our gossypetin dose regimen. To assure the stability of gossypetin in various biological matrix, we investigated the stability of gossypetin in PBS, mouse plasma, and mouse LM. The results showed that gossypetin was stable in the PBS and plasma but metabolized in LM with a half-life of 2.43 ± 0.48 h (Fig. 7E). Using the slope (K in Fig. 7D) calculated from the metabolic stability results and in vivo scaling factor such as LM concentration, protein content, and hepatic blood flow, hepatic Clint and ER was calculated as 74.9 ± 13.8 mL/min/kg and 0.45 (0.41, 0.50), respectively (Fig. 7E) (Huang et al., 2015). Hepatic extraction ratio values of <0.25, 0.25-0.75, and >0.75 represent low, moderate, and high extraction compounds, respectively (Kim et al., 2020). It suggested that gossypetin undergoes moderate hepatic metabolism, which may explain the relatively long plasma half-life after the oral administration of gossypetin at a dose of 20 mg/kg.

4. Discussion

NASH is severe liver metabolic disorders increasing in incidence due to lifestyle changes (Utzschneider and Kahn, 2006; Wree et al., 2013; Younossi et al., 2018). Despite the increasing prevalence of NASH, there is currently no FDA-approved drug for its treatment due to the multiple complex mechanisms involved in its etiology(Takaki et al., 2013). Previously, many of studies and the drug development for NASH were based on the one-hit hypothesis. However, the development of therapeutic agents that target one specific mechanism to improve NASH
pathology has failed. As a result, the drug development approach for NASH has shifted to a newly established "multiple-hit hypothesis," which considers targeting various factors simultaneously to increase the effectiveness of therapeutic agents. According to this hypothesis, multiple drugs must be administered in combination therapy to enhance efficacy (Vuppalanchi et al., 2021), however, this strategy can potentially lead to undesirable side effects. To solve this problem, we attempted to identify a single drug that targets multiple causes of NASH.

Targeting metabolic dysfunction and oxidative stress could aid in the prevention of NASH pathology since those two factors are considered as the inducers of chronic inflammation and fibrosis in pathological conditions. When the high-fat or high-sugar diets are consumed over a long time, insulin resistance and lipid metabolic dysfunction are induced in adipose and liver tissue (Qureshi and Abrams, 2007). Dysregulated metabolic signals produce excessive fatty acids and it can further activate systemic immune cells by a number of adipokines and chemokines from adipose tissue (Balistreri et al., 2010). Activated immune cells can infiltrate into the liver tissue and secrete pro-inflammatory cytokine, which may increase the possibility of several disease progression (Turner et al., 2014). Excessive fatty acids also produce reactive oxygen species (ROS) in the mitochondria which may further increase the inflammation in the liver tissue. ROS can dissociate the complex of thioredoxin (TRX) and thioredoxin-interacting protein (TXNIP) (Zhou et al., 2010). In this stage, the dissociated TXNIP binds to NLRP3, which is an inflammasome factor that triggers the production of pro-inflammatory cytokine (Zhao et al., 2020; Zhou and Chng, 2013). In addition, oxidative stress directly or indirectly regulates the expression of TGF-β in the various cells and induces fibrosis by activating hepatic stellate cells (Hiraga et al., 2013; Lin et al., 2010; Montorfano et al., 2014). Likewise, targeting oxidative stress through antioxidants, like vitamin E, improves the liver toxicity, inflammation, and fibrosis during human clinical study (Sato et al., 2015). Together with, it has been reported that antioxidants like vitamin E improve liver toxicity, inflammation, and fibrosis on human clinical study by reduction of oxidative stress (Sato et al., 2015). In this study, gossypetin also shows inhibitory effect on hydrogen peroxide- and palmitate-induced
oxidative stress in AML12 cells and lipid peroxidation in the liver tissue of NASH model mice improving their NASH pathology. It supports the idea that antioxidants have possibility to alleviate NASH. AMPK is also an attractive therapeutic target to improve metabolic dysfunction in the NASH pathological condition. AMPK is one of serine/threonine kinases, that is activated in low energy level and play a role as an energy sensor by detecting the ratio of AMP/ATP(Gowans and Hardie, 2014). AMPK mainly phosphorylates acetyl-CoA carboxylase 1 (ACC), which is a key enzyme for fatty acid synthesis and it regulates hepatic insulin resistance(Fullerton et al., 2013). When the AMPK is activated specifically in the liver tissue, liver steatosis is decreased in a high-fructose diet-induced mice model(Woods et al., 2017). In addition, genetically induced mice model of AMPK phosphorylation shows inhibitory effects to gene expression related to inflammation and fibrosis(Garcia et al., 2019). Through our results, we showed that gossypetin is an outstanding antioxidant and a novel AMPK activator that improves the pathologies of NASH. Based on these evidence, double targeting action of gossypetin could be an effective treatment for NASH.

Previous studies have reported the alleviating effects of flavonoid metabolites on the pathology of NAFLD and NASH (Tan et al., 2022). Among these flavonoids, quercetin is considered a promising therapeutic candidate due to its ability to improve liver inflammation and fibrosis(Li et al., 2018). Additionally, it has been observed that quercetin reduces lipid dysregulation in NAFLD by modulating the AMPK activity (Gnoni et al., 2022). To compare the effect on AMPK phosphorylation level between other flavonoids and gossypetin, we treated those flavonoids to AML12 cells. Our results revealed that gossypetin induced higher AMPK phosphorylation compared to the other flavonoids (Supplementary Figure 11). This suggests that gossypetin is more effective in activating AMPK than other flavonoids including quercetin.

The most widely used direct AMPK activator by far is 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR). Although, it has been reported to have a preventive effect on liver steatosis, it suffers from poor selectivity and rapid clearance in vivo system (Feng et al., 2018).
The elimination half-life (T_1/2) of AICAR after its intravenous administration at a dose of 200 mg/kg was 0.44 h in mice (Cheng et al., 2013). Furthermore, AICAR tends to accumulate in cells at millimolar concentrations through adenosine transporter leading to AMPK-independent or off-target effects, which hinders its use as a specific AMPK activator (Gadalla et al., 2004). Another well-known AMPK activator is A-769662, which has been developed as a selective ADaM site-binding activator for improving metabolic dysfunction (Cool et al., 2006b). However, studies have shown that A-769662 inhibits proteasome 26S activity independent AMPK signaling pathway and exhibits off-target toxicity in mouse embryonic fibroblasts (García-García et al., 2010; Moreno et al., 2008; Zadra et al., 2014). Additionally, A-769662 showed low clearance and low oral bioavailability (Feng et al., 2018). Furthermore, it has been reported that A-769662 can directly inhibit the sodium pump in skeletal muscle, independent of AMPK activation (Benziane et al., 2009). These different actions challenge the application of A-769662 in clinical application for metabolic disorders. When we compared to the AMPK binding affinity between gossypetin or A-769662 using an in silico docking analysis, the binding energy and affinity appeared to be similar (Supplemental Fig. 7E-G). Moreover, gossypetin induced greater AMPK phosphorylation than both AICAR and A-769662, as shown in Figure 5, and exhibited a similar EC_{50} as A-769662 (Cool et al., 2006a). From a pharmacokinetic perspective, gossypetin is an ideal candidate for treating NASH. When comparing the pharmacokinetic properties of gossypetin, it was found to be stably detectable in plasma after oral administration at an effective concentration (20 mg/kg), with a T_{1/2} of 8.9±2.4 h. Additionally, distribution of gossypetin was favorable to the liver, the target organ of this study and maintained the concentrations over EC_{50} value for AMPK activation. Conversely, gossypetin showed similar or lower distribution to other tissues such as the brain, kidney, heart, muscle, and adipose tissue, suggesting preferential activation of AMPK in the liver. Furthermore, when comparing the anti-NASH effects of gossypetin to those of AICAR or A-769662 using a mouse model of MCD diet-induced NASH, gossypetin demonstrated a superior preventive effect (Supplemental Fig. 8). Importantly, gossypetin did not exhibit the previously
reported off-target effects associated with well-known AMPK activators, further indicating its effectiveness and safety compared to other agents (Supplemental Fig. 9).

Unlike A-769662 and AICAR, gossypetin is a natural compound that has an additional antioxidant effect, and thus, it could potentially be safer. To ensure the toxicity of gossypetin, we measured the body weight and the tissue weight after the treatment of gossypetin to MCD diet-induced mice model. There were no changes to the weight of the tissues from gossypetin-treated mice (Supplemental Fig. 3). Although additional toxicity tests are required, the safety of gossypetin could be expected based on these results. For further study, we will thoroughly evaluate toxic effect of gossypetin which will be necessary for its application into clinical study targeting NASH patients.

**Author Contribution**

Participated in research design: Eunji Oh, Kyong-Tai Kim

Conducted experiments: Eunji Oh, Jae Lee, Sungji Cho, Sung Wook Kim, Kyung Won Jo, Seung Hee Gwak, So Yeon Jeon, Jin-Hyang Park, Im-Sook Song, Themis Thoudam

Contributed new reagents or analytic tools: Eunji Oh, Won Sik Shin, Im-Sook Song, Seonyong Kim, Joohun Ha

Performed data analysis: Eunji Oh, Jae Lee, Sungji Cho, Sung Wook Kim, Won Sik Shin, Kyung Won Jo, Im-Sook Song, Themis Thoudam, In-Kyu Lee, Seonyong Kim, Se-Young Choi, Kyong-Tai Kim

Wrote or contributed to the writing of the manuscript: Eunji Oh, Kyong-Tai Kim
Reference


FOOTNOTES

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Conflicts of interest

The authors declare no conflict of interest.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Figure legend

Fig. 1. Gossypetin exerted preventive effects against nonalcoholic steatohepatitis (NASH) pathology in methionine–choline-deficient (MCD) diet-fed mice.

(A) Experimental design of the MCD diet-fed mouse experiment. (B, C) MCD diet feeding decreased body and liver tissue weight. Gossypetin did not affect body or liver weight. (D) Aspartate aminotransferase and alanine aminotransferase levels in serum samples (n = 17–20). (E) Representative hematoxylin and eosin (H&E). H&E staining revealed NASH pathology featuring steatosis, lobular inflammation (black arrow), and hepatocyte ballooning (red arrow). (F, G) Quantified nonalcoholic fatty liver disease activity score (NAS). (H, I) Representative oil red O staining images. Oil red O staining-positive area was measured using ImageJ. (J) Free fatty acid contents in the liver tissue (n=9-10). (K) Quantitative PCR data of lipogenic genes (Srebp1c and Acox1) and genes related to β-oxidation process (Cpt1α and Acox1). Data represent the mean ± standard error of the mean. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way analysis of variance.

Fig. 2. Gossypetin alleviated the pathology of chronic inflammation and fibrosis in the liver tissue of methionine–choline-deficient (MCD) diet-fed mice.

(A, B) Representative immunohistochemical images of staining for F4/80, a marker of infiltrated macrophages, in the liver tissue of MCD diet-fed mice treated with vehicle or gossypetin. (C) Quantitative PCR data of inflammatory response factors (Mcp-1 and Cd68). (D, E) Representative images and quantification of Sirius red staining illustrating collagen deposition. (Magnification, ×20; scale bar = 100 μm and 200 μm). (F) Total collagen content was examined by measuring hydroxyproline levels (n = 17–20). (G) Quantitative PCR data of the expression of collagen components (Col1α and Col3α). Data represent the mean ± standard error of the mean. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way analysis of variance.

Fig. 3. Gossypetin decreased hydrogen peroxide-induced oxidative stress in AML12 cells

(A) The 2,2-diphenyl-1-picrylhydrazyl assay was used to assess the radical-scavenging activity of gossypetin. Ascorbic acid was used as positive control. (B) 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) assay. Trolox was used as positive control. (C, D) Gossypetin suppressed hydrogen peroxide-induced oxidative stress. Cells were treated with hydrogen peroxide (10 mM) for 2 h after 24 h of gossypetin pretreatment. The level of oxidative stress was quantified by the intensity of green fluorescence using fluorescence microscopy. (E, F) AML12 cells were treated with palmitate-conjugated bovine serum albumin (450 μM) after the treatment with gossypetin for 24 h. The level of oxidative stress was quantified through the
intensity of green fluorescence using fluorescence microscopy. (G, H) Representative images of dihydroethidium staining to reveal oxidative stress in the liver tissue of methionine–choline-deficient (MCD) diet-fed mice (n = 8–10). All data are presented as the mean ± standard error of the mean. **p < 0.01, ***p < 0.001 by one-way analysis of variance or t-test.

**Fig. 4. Gossypetin prevents oxidative stress-induced mitochondrial dysfunction and hepatic cell death**

(A, B) Oxygen consumption rate was measured following the treatment with palmitate (250 μM) along with gossypetin (25 μM) or DMSO. (C, D) Mitochondrial membrane potential (MMP) was measured through TMRE staining experiment. Mitochondria dysfunction was induced by the treatment with palmitate (PA; 100 μM) for 24 h. Fluorescence intensity was measured using ImageJ program. (E, F). Representative immunohistochemical staining of cleaved caspase-3, a marker of hepatic cell death, in the liver tissue of MCD diet-fed mice treated with either vehicle or gossypetin (n = 7–10). All data are presented as the mean ± standard error of the mean. **p < 0.01, ***p < 0.001 by two-way analysis of variance or one-way analysis of variance.

**Fig. 5. Gossypetin induces AMP-activated protein kinase (AMPK) phosphorylation via direct binding to AMPK.**

(A, B) Representative western blot data to confirm the phosphorylation level of AMPK in AML12 cells. EC₅₀ was measured using the PRISM GraphPad program. (C, D) Representative western blot data to confirm the phosphorylation level of AMPK in the liver lysate of methionine–choline-deficient (MCD) diet-fed mice. (E, F) Representative Western blot data that compare the phosphorylation level of AMPK by gossypetin to the known AMPK activators (AICAR and A-769662) (G) Molecular docking analysis, docking positions, and binding energy of gossypetin to AMPK subunit (code: 5iso) were predicted using PyRx and PyMol software. (H) CNBr bead pulldown assay. Gossypetin was incubated with CNBr beads for 24 h to permit conjugation, and the cellular lysate of AML12 cells was incubated with the CNBr beads. All experiments were repeated at least three times. Data are presented as the mean ± standard error of the mean. **p < 0.01, ***p < 0.001 by one-way analysis of variance or t-test.

**Fig. 6. Gossypetin decrease lipid accumulation in the AML12 cells depends on AMPK activation.**

(A, B) Representative oil red O staining images. Oil red O staining-positive area was measured using ImageJ. Compound C (5 μM) was treated to inhibit AMPK phosphorylation for 24 hr to AML12 cells. (C, D) Representative Western blot data to confirm the effect of gossypetin on
AMPK phosphorylation in AML12 cells. (E, F) Representative Western blot data to confirm the effect of gossypetin on ACC phosphorylation in AMPK α1/2 double KO MEF.

**Fig. 7. Pharmacokinetics and tissue distribution of gossypetin.**

(A) Plasma and liver concentration-time profile of gossypetin in mice following single oral administration of gossypetin (20 mg/kg). (B) Tissue concentration time profile of gossypetin in mice following single oral administration of gossypetin (20 mg/kg). (C) Pharmacokinetic parameters of gossypetin. All data are presented as the mean ± standard deviation (n=6). Ratio of gossypetin in tissue to gossypetin in plasma (T/P) was calculated from AUC<sub>tissue</sub>/AUC<sub>plasma</sub>. * p<0.05; ** p<0.01 compared with plasma gossypetin and expressed as mean T/P ratio (95% confidence intervals). (D) Stability profile and (E) stability parameters of gossypetin in PBS, mouse plasma, and LM was measured for 2 h. All data are presented as the mean ± standard deviation (n=4). Elimination slope (K) was calculated from the log transformed gossypetin amount vs time profile, Half-life was calculated from Ln2/K. Hepatic intrinsic clearance (Clint), and hepatic extraction ratio (ER) were calculated according to the following equation in method section. ER was expressed as mean ER (95% confidence intervals). * p<0.05; ** p<0.01 compared with gossypetin stability in PBS.
Figure 3

A. 

DPPH

- Ascorbic Acid
- Gossypetin

Concentration (μM)

B. 

ABTS

- Trolox
- Gossypetin

Concentration (μM)

C. 

Hydrogen peroxide

Control  DMSO  Gossypetin (25 μM)

Cellrox

DAPI

D. 

%Area

Control  H₂O₂  H₂O₂ + Gossypetin

E. 

Palmitate

Control  DMSO  Gossypetin (25 μM)

Cellrox

DAPI

F. 

%Area

Control  PA  PA + Gossypetin

G. 

MCD  MCD + Gossypetin

DHE

DAPI

H. 

DHE Fluorescent Intensity (pmol/h)

MCD  MCD + Gossypetin

MCD + Ven

MCD + Ven + Gossypetin
Figure 6

A. 

![Image of cell cultures with various treatments](image)

B. 

![Bar graph showing relative values](image)

C. 

<table>
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<th>Phospho-AMPK</th>
<th>Total AMPK</th>
<th>GAPDH</th>
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<td>DMSO</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gossypetin</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Compound C</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Compound C + Gossypetin</td>
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D. 

![Bar graph showing relative AMPK level](image)

E. 

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<th>GAPDH</th>
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<tr>
<td>AMPK α1/2 KO</td>
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<td>-</td>
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<tr>
<td>Gossypetin</td>
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F. 

![Bar graph showing relative ACC level](image)
Figure 7

A. Concentration (nM) of gossypelin over time for plasma and liver samples. The EC_{so} is 0.43 μM.

B. Concentration (nM) of gossypelin over time for various organs (brain, kidney, heart, muscle, adipose, and plasma).

C. Pharmacokinetic parameters for plasma and liver samples:

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<th>Liver</th>
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<td>656.7±241.7 **</td>
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<td>917.3±309 *</td>
</tr>
<tr>
<td>AUC_{4h} (nM·h)</td>
<td>649.7±175</td>
<td>1013±242 *</td>
</tr>
<tr>
<td>T_{1/2} (h)</td>
<td>8.9±2.4</td>
<td>1.2±0.8 **</td>
</tr>
<tr>
<td>T/P</td>
<td>1.73</td>
<td></td>
</tr>
</tbody>
</table>

D. Gossypelin % remaining over time for different conditions:

- PBS
- Mouse plasma
- Mouse liver microsomes

E. Pharmacokinetic parameters for PBS, plasma, and LM:

<table>
<thead>
<tr>
<th>Condition</th>
<th>% remaining after 2 h</th>
<th>K (h⁻¹)</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>95.4±7.1</td>
<td>0.035</td>
<td>19.8</td>
</tr>
<tr>
<td>Plasma</td>
<td>92.3±5.6</td>
<td>0.043</td>
<td>16.1</td>
</tr>
<tr>
<td>LM</td>
<td>57.8±4.7</td>
<td>0.285</td>
<td>2.43</td>
</tr>
</tbody>
</table>