European Journal of Pharmacology 727 (2014) 115–124

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

SIRT1 activation by methylene blue, a repurposed drug, leads to AMPK-mediated inhibition of steatosis and steatohepatitis

Seo Young Shin [a,1], Tae Hyun Kim [a,1], Hongmin Wu [a], Young Hee Choi [b], Sang Geon Kim [a,*]

[a] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, South Korea
[b] College of Pharmacy, Dongguk University, 32 Dongguk-lo, Ilsan dong-gu, Goyang, Gyeonggi-do 410-820, South Korea

ARTICLE INFO

Article history:
Received 11 October 2013
Received in revised form
13 January 2014
Accepted 22 January 2014
Available online 30 January 2014

Keywords:
Methylene blue
Steatoheptitis
Mitochondria
Lipogenesis
SIRT1

ABSTRACT

Sirtuins maintain energy balance. Particularly, sirtuin 1 (SIRT1) activation mimics calorie restriction and nutrient utilization. However, no medications are available for the up-regulation of SIRT1. Methylene blue (MB) had been in clinical trials for the treatment of neurological diseases. This study investigated the effect of MB on sirtuin expression in association with the treatment of steatosis and steatohepatitis, and explored the underlying basis. The effects of MB on mitochondrial function, molecular markers, pharmacokinetics, and histopathology were assessed using hepatocyte and/or mouse models. Immunoblotting, PCR and reporter assays were done for molecular experiments. After oral administration, MB was well distributed in the liver. MB treatment increased NAD^+/NADH ratio in hepatocytes. Of the major forms, MB treatment up-regulated SIRT1, and thereby decreased PGC-1α acetylation. Consistently, hepatic mitochondrial DNA contents and oxygen consumption rates were enhanced. MB treatment also notably activated AMPK, CPT-1 and PPARα: the AMPK activation relied on SIRT1. Activation of LXRα and the induction of SREBP-1c and its target genes by T0901317 were diminished by MB. In addition, MB treatment antagonized the ability of palmitate to acetylate PGC-1α, and increase SERBP-1c, FAS, and ACC levels. In mice fed on a high-fat diet for 8 weeks, MB treatment inhibited excessive hepatic fat accumulation and steatohepatitis. The ability of MB to activate SIRT1 promotes mitochondrial biogenesis and oxygen consumption and activates AMPK, contributing to anti-lipogenesis in the liver. Our results provide new information on the potential use of MB for the treatment of steatosis and steatohepatitis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the hepatic constituent of metabolic syndrome, and may result in hepatitis and/or facilitate the progression of more severe liver diseases such as fibrosis and cirrhosis (Marra et al., 2008). Aberrant hepatic fat accumulation is provoked mainly by decreases in mitochondrial fuel oxidation and increases in liver X receptor α (LXRα)-dependent lipogenesis. Excessive fat accumulation and fatty acid oxidation within hepatocytes cause oxidative stress in the liver and promote the production of proinflammatory cytokines (Mantena et al., 2008, 2009). The consequent oxidative stress triggers a robust production of lipid peroxides that form adducts to macromolecules, and chronically results in damage to organelles such as mitochondria (Marra et al., 2008).

Sirtuin (SIRT) maintains energy balance in the cell (Rodgers et al., 2008); the activation of SIRT mimics several metabolic aspects of calorie restriction that enhance selective nutrient utilization and mitochondrial oxidative function. A line of studies support the role of SIRT1 in homeostatic energy metabolism under metabolic disorders including fatty liver, insulin resistance, and atherosclerosis; SIRT1 regulates peroxisomal proliferators-activated receptor alpha (PPARα) and PPARγ coactivator (PGC-1α), the molecules that govern mitochondrial fuel oxidation and lipid homeostasis (Lagouge et al., 2006). Since SIRT1 is the key molecule that activates AMPK (Lan et al., 2008), it may be an attractive target for the treatment of metabolic disorders. However, no medications are currently available for the induction of SIRT1.

Methylene blue (MB) regulates electron transfer chain reaction within the mitochondria, and enhances mitochondrial respiration by exchanging electron at complex I and increasing the activity of

Abbreviations: ACC, acetyl CoA carboxylase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltrasnferase-1; FAS, fatty acid synthase; FFA, free fatty acid; HFD, high-fat diet; LKB1, liver kinase B1; MB, methylene blue; LXRα, liver X receptor-α; LXRE, LXRα response element; mtDNA, mitochondrial DNA; mtCOX II, mitochondrial cytochrome *c* oxidase subunit II; NAFLD, non-alcoholic fatty liver disease; NAD^+, nicotinamide adenine dinucleotide; ND, normal diet; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1α; PPARα, peroxisome proliferator-activated receptor α; RIP140, receptor-interacting protein 140; SREBP-1c, sterol regulatory element binding protein-1c; TG, triglyceride; T090, T0901317

* Corresponding author. Tel.: +82 2 880 7840; fax: +82 2 872 1795.
E-mail address: sgk@snu.ac.kr (S.G. Kim).
[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.ejphar.2014.01.035

cytochrome *c* oxidase (Wen et al., 2011). So, MB has been utilized as a redox indicator. Recently, MB has been in clinical trials for the treatment of pathologic conditions such as methemoglobinemia, ischemic-reperfusion injury, and cyanide poisoning (Wen et al., 2011). Moreover, it may also be applied for the treatment of Alzheimer disease, a disease highly associated with mitochondrial dysfunction (Atamna et al., 2008; Eckert et al., 2012); MB treatment protects from the cognitive decline inflicted by inhibitors of complex IV through its redox activity (Callaway et al., 2002). Nevertheless, the effect of MB on sirtuin expression had never been explored.

This study investigated whether MB has an effect on sirtuin expression, and if so, whether it has therapeutic efficacy for the treatment of NAFLD. To accomplish this, we used in vitro and in vivo models (i.e., hepatocyte-derived cell line; primary rat hepatocyte; mouse pharmacokinetics; and feeding of a high fat diet, HFD). Here, we report that MB has the ability to activate SIRT1 and promote mitochondrial biogenesis and oxidation of free fatty acids (FFAs). Our findings demonstrate the beneficial effect of MB on metabolic homeostasis in conjunction with the inhibition of liver fat accumulation, implying that MB may be utilized as a repurposed drug for the treatment of steatosis and steatohepatitis.

2. Materials and methods

2.1. Materials

MB and methylene violet [internal standard (IS) for ultra-performance liquid chromatography tandem mass-spectrometric (UPLC-MS/MS)] were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Acetonitrile and heptafluorobutyric acid were obtained from Fisher Scientific Co. (Seoul, Korea), and all other chemicals and reagents used for pharmacokinetic studies were of analytical grade. T0901317 (T090) was obtained from Calbiochem (San Diego, CA, USA). The antibodies recognizing SREBP-1, PPARα, and PGC-1α were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas those directed against p-AMPK, AMPK, p-ACC, ACC, and SIRT3 were from Cell Signaling Technology (Beverly, MA, USA). An anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA). Anti-SIRT1 and anti-SIRT5 antibodies were provided from Millipore (Billerica, MA, USA) and Abcam (Cambridge, MA, USA), respectively. The antibody directed against mouse CPT1 was obtained from Proteintech (Chicago, IL, USA). GW3965 and compound C were supplied from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Calbiochem (Darmstadt, Germany), respectively. Horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat, and goat anti-mice IgGs were obtained from Zymed Laboratories (San Francisco, CA, USA).

2.2. NAD^+/NADH assay

The NAD^+/NADH ratio in liver homogenate or cell lysate was measured using commercially available kit (Abcam, Cambridge, MA, USA).

2.3. Animal treatment

Animal studies were conducted in accordance with the institutional guidelines for the care and use of laboratory animals, and were approved by the internal committees of the universities. Male C57BL/6 mice (Charles River Orient, Seoul, Korea) were acclimatized for 1 week at the Animal Center for Pharmaceutical Research, Seoul National University. To assess molecular changes, mice were treated with MB dissolved in tap water (3 or 10 mg/kg/day, p.o.) for 3 days, and were sacrificed 12 h after the last dose. In addition, mice at 6 weeks of age were started on either a normal diet (ND) or HFD (fat 60% w/w, Dyets Inc., Bethlehem, PA, USA) for 8 weeks. After 4 weeks, the HFD-fed mice were distributed into three treatment groups ($n=9$–10 each). MB was orally administered to mice four times per week during last 4 weeks of HFD feeding. Control animals received vehicle only.

2.4. Pharmacokinetic studies

In pharmacokinetic studies, MB (dissolved in distilled water) was administered at a dose of 3 or 10 mg (5 ml)/kg via the jugular vein or orally using a gastric gavage tube. The micro-blood sampling system was programmed to collect a 10 μl aliquot of blood, and samples were collected at designated times via the carotid artery. Urine and the entire gastrointestinal tract (including its contents and feces) were collected at 24 h. In tissue distribution studies, all procedures were done in the same way. After oral administration of MB, blood was collected as much as possible via the carotid artery, and each liver, spleen, intestine, kidney, fat, lung, heart, spleen, muscle and brain were excised and homogenized with a four-fold volume of distilled water. Other procedures used were similar to the reported method (Singh, 2006; Choi et al., 2010; Han et al., 2013).

MB in all biological samples was analyzed on a Waters UPLC-XEVO TQ-S system (Waters Corporation, Milford, MA, USA). Chemicals were separated on a reversed-phase C_{18} column (ACQUITY UPLC BEH C_{18}, 2.1 mm × 100 mm i.d., 1.7-μm particle size; Waters, Ireland) with a flow rate of 0.3 ml/min. The compositions of mobile phase, 5 mM heptafluorobutyric acid (A) and acetonitrile (B), were 45:55 (v/v). The MS was operated in the multiple reaction monitoring mode with electrospray ionization interface used to positive ions ($[M-H]^+$) at a capillary voltage of 1.0 kV, a source temperature of 150 °C and a desolvation temperature of 350 °C. Cone voltage and collision energy were optimized for MB and IS. The mass transitions used for MB and IS were m/z 283.90→268.11 (50 and 40 eV for cone voltage and collision energy, respectively) and 343.3→299.6 (40 and 38 eV, respectively), respectively. A 100 μl of acetonitrile containing 500 ng/ml IS was added to a 50 μl aliquot of mouse plasma sample, and then 10 μl of the supernatant was directly injected into the column. The retention times of MB and IS were approximately 1.3 and 2.3 min, respectively. Standard methods (Gibaldi and Perrier, 1982) were used to calculate the pharmacokinetic parameters using a non-compartmental analysis (WinNonlin 2.1; Pharmasight Corp., Mountain View, CA, USA). The extent of absolute oral bioavailability (F) was calculated from the ratio of AUC_{oral}/AUC_{iv}.

2.5. Cell culture

HepG2 and AML-12 cell lines were purchased from ATCC (Manassas, VA, USA). The cells were plated at 1×10^5 per well in six-well plates, and wells with 70–80% confluency were used. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, whereas AML-12 cells were grown in a 1:1 mixture of DMEM supplemented with insulin-transferrin-selenium-X (ITSX; Gibco®) and dexamethasone (40 ng/ml; Sigma), and 10% fetal bovine serum. Total cell lysates and nuclear extracts were prepared as previously described (Hwahng et al., 2009).

2.6. Assays on mitochondrial DNA and marker proteins

Total DNA was extracted from tissues according to standard procedures. mtDNA was determined by quantitative real-time PCR (qRT-PCR) using SYBR green; fluorescence intensities were