Identification of bioactive heterocyclic compounds from mulberry and their protective effect against streptozotocin-induced apoptosis in INS-1 cells

JI HOON SONG1, DAHAEE LEE2,3, SEOUNG RAK LEE3, JAE SIK YU3, TAE SU JANG4, JOO-WON NAM5, KI HYUN KIM3 and KI SUNG KANG2

1Department of Medicine, University of Ulsan College of Medicine, Seoul 05505; 2College of Korean Medicine, Gacheon University, Seongnam, Gyeonggi 13120; 3School of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi 16419; 4Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang, Gangwon 25354; 5College of Pharmacy, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Republic of Korea

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Abstract. A phytochemical investigation of the MeOH extracts from mulberries (the fruit of Morus alba L.) led to the identification of six heterocyclic compounds (1-6). These compounds were screened to determine whether they protected pancreatic INS-1 cells from streptozotocin (STZ)-induced cytotoxicity. Compound 3 was the most effective at preventing STZ-induced cytotoxicity and the production of reactive oxygen species (ROS) in INS-1 cells. In addition, compound 3 effectively prevented apoptosis induced by STZ in INS-1 cells. Compound 3 also prevented STZ-mediated cleavage of caspase-8, caspase-3 and poly (ADP-ribose) polymerase and increased the expression of B-cell lymphoma-2 (Bcl-2), an anti-apoptotic Bcl-2 family protein. In conclusion, the results of the present study indicate that compound 3 extracted from the fruit of M. alba was highly effective in preventing type 1 diabetes mellitus and may be a novel treatment option.

Introduction

Diabetes mellitus, mainly caused by various genetic and environmental factors, is a chronic and systemic metabolic syndrome. Diabetes results from a shortage in the amount of insulin released from the pancreas in response to elevated blood glucose or from a deficiency in the ability of fat and muscle cells to respond to insulin (1). The representative symptoms of diabetes are increased hunger, frequent urination, and increased thirst, and untreated diabetes causes a variety of complications including chronic kidney disease, stroke, cardiovascular disease, and diabetic retinopathy (1-3). Type 1 diabetes, also called insulin-dependent diabetes mellitus (IDDM), occurs via an autoimmune reaction that attacks the β cells of the pancreas that produce insulin (4). Type 2 diabetes, or non-insulin-dependent diabetes (NIDDM), is caused by a deficiency in the insulin-responsive system, and a strong connection between type 2 diabetes and obesity has become clear (5). Currently, there are many different classes of anti-diabetic drugs used in clinical practice to decrease blood glucose levels, such as metformin, glucagon-like peptide-1 receptor (GLP-1) agonists, and synthetic insulin analogs (2,6). Nevertheless, many studies on natural products are being carried out to discover potent anti-diabetic lead compounds that have minimal side effects.

Morus alba L. (Moraceae), known as the white mulberry tree, is cultivated in Asia, Europe, and India, and its fruits, commonly known as mulberry, are widely cultivated as an edible fruit (7). Mulberry has been used as a traditional medicine in East Asia for the prevention of insomnia, dizziness, and tinnitus as well as the alleviation of high glucose levels (8,9). Previous researches have reported that various chemical constituents and extracts from this natural source exhibit useful pharmacological activities including anti-inflammatory, antioxidant, and immunoregulatory effects (10-12). A recent study reported that anthocyanin-rich mulberry extracts alleviate high glucose levels in in vivo studies of glucose consumption and uptake, which was attributed to AMPK/ACC/mTOR signaling (13). In addition, anti-hyperglycemic and anti-hyperlipidemic effects of polysaccharides from the fruits of M. alba have been reported, which provide a scientific rationale for the development of this source as a new medication candidate to treat diabetes (14). However, anti-diabetic compounds/metabolites from M. alba
fruits have not yet been fully investigated. The present study describes the protective effects of compounds isolated from *M. alba* fruits against STZ-induced INS-1 cell death as well as its molecular mechanisms in the apoptotic pathway.

**Materials and methods**

*Extraction of M. alba fruits and isolation method.* The fruits of *M. alba* were bought at the Kyungdong Market (Woori Herb, Seoul, Korea, in January, 2014. A voucher specimen (MA 1414) of the material was classified by one of the authors (K.H. Kim) and was stored in the herbarium of the School of Pharmacy, Sungkyunkwan University (Suwon, Korea). Dried and pounded fruits of *M. alba* (10.0 kg) were extracted with 70% aqueous MeOH three times at room temperature and then filtered. The filtrate was condensed *in vacuo*, affording a slurry resultant (1.4 kg). The resultant residue was dissolved in deionized water and successively partitioned with hexane, CHCl3, EtOAc, and n-BuOH (800 ml x3) until the color of partitioned layer disappears, providing 27.8, 85.3, 32.9, and 138.8 g, respectively. The CHCl3-soluble fraction (85.0 g) was loaded to a silica gel (230-400 mesh) column and fractionated using CHCl3-MeOH (40:1-1:1, gradient system) to yield five fractions (CA-CE). Fraction CB (4.3 g) was separated by RP-C18 silica gel (230-400 mesh) column chromatography eluted with 70% MeOH/H2O to give eleven fractions (CB1-CB11). Fraction CB1 (226 mg) was passed over Sephadex LH-20 column chromatography eluted with 100% MeOH to give six subfractions (CB1-1-CB1-6). Subfraction CB1-3 (33 mg) was purified by semi-preparative reversed-phase HPLC using an isocratic solvent system of 4% MeOH/H2O (Phenomenex Luna Phenyl-hexyl, 250x10.0 mm, 5 µm, flow rate: 2 ml/min) to afford compounds 1 (0.4 mg, tR=25.0 min) and 2 (4.4 mg, tR=37.2 min). Fraction CB2 (750 mg) was fractionated using silica gel (230-400 mesh) column chromatography eluted with CHCl3-MeOH (40:1-5:1, gradient system) to afford nine subfractions (CB2-1-CB2-9). Subfraction CB2-2 (176 mg) was separated by semi-preparative reversed-phase HPLC using an isocratic solvent system of 29% MeOH/H2O (Phenomenex Luna Phenyl-hexyl, 250x10.0 mm, 5 µm, flow rate: 2 ml/min) to yield compounds 3 (2.1 mg, tR=72.0 min) and 4 (1.6 mg, tR=65.1 min). Subfraction CB2-3 (84 mg) was also separated by semi-preparative reversed-phase HPLC using an isocratic solvent system of 18% MeOH/H2O (Phenomenex Luna Phenyl-hexyl, 250x10.0 mm, 5 µm, flow rate: 2 ml/min) to afford compound 5 (1.3 mg, tR=40.5 min). Finally, fraction CB4 was separated on silica gel (230-400 mesh) column chromatography using CHCl3-MeOH (40:1-5:1, gradient system) to give seven subfractions (CB4-1-CB4-7). Compound 6 (1.8 mg, tR=26.1 min) was purified from subfraction CB4-2 (20.0 mg) by utilizing semi-preparative reversed-phase HPLC with an isocratic solvent system of 58% MeOH/H2O (Phenomenex Luna Phenyl-hexyl, 250x10.0 mm, 5 µm, flow rate: 2 ml/min).

*Cell culture.* INS-1 cell line, immortalized rat pancreatic islet beta cells, were purchased from Biohermes (Shanghai, China) and grown in RPMI-1640 (Cellgro, Manassas, VA, USA) supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen Co., Grand Island, NY, USA), 11 mM d-glucose, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol in an humidified atmosphere supplying of 5% CO2 at 37°C.

*Measurement of the level of intracellular ROS.* The levels of intracellular ROS were measured using 2’,7’-dichlorodihydrofluorescein diacetate (H2-DCF-DA; Sigma, St Louis, MO, USA). Cells were plated into clear bottomed 96-well black plates at a density of 2×104 cells per well and adhered for 24 h. After the pre-treatment with control or indicated concentrations of compounds for 2 h, cells were then exposed to 50 µM streptozotocin (STZ; Sigma) for additional 24 h. After incubation, cells were stained 10 µM H2DCFDA for 30 min followed by washing with PBS three times. Green DCF fluorescent intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Ex/Em) using a fluorescent microplate reader (Tecan Infinite F200 Microplate Fluorescence Reader; Tecan Zürich, Switzerland).

*Assessment of cell viability.* Cell viability were determined using Ez-Cytox cell viability detection kit following manufacturer’s instruction. In brief, cells were grown in 96-well plate at a density of 2×104 cells per well for 24 h. Cells were then pre-treated with control (0.5% DMSO) or the indicated concentrations of compounds. After incubation for 2 h, cells were exposed to 50 µM STZ for 24 h. Cells were then incubated with 10 µl Ez-Cytox for additional 2 h. Cell viability was determined from the absorbance at 450 nm using a microplate reader.

*Tali-image based analysis of apoptotic cells.* Cells were plated in 6-well plates at a density of 3×105 cells per well and incubated for 24 h to adhere. Cells were pre-treated with 50 and 100 µM compound 3 for 2 h and exposed to 50 µM STZ for 24 h. The cells were then harvested and washed with PBS.
Cells were incubated with an Annexin V Alexa Fluor 488 in Annexin-binding buffer for 20 min followed by staining with propidium iodide (PI). The percentage of apoptotic cells was analyzed using a Tali image-based cytometer (Invitrogen, CA, USA). In this analysis, the apoptotic cells were determined by the percentage of Annexin V-positive cells on total counted-cells.

Western blot analysis. Cells were plated in 6-well plates at a density of 3x10^5 cells per well and incubated for 24 h to adhere. Cells were pre-treated with 50 and 100 µM compound 3 for 2 h and exposed to 50 µM STZ for 24 h. Cells were then lysed with RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride immediately before use. The equal amounts of protein were separated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies against cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, B-cell lymphoma-2 (BCL-2), PARP, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were detected using ECL Advance western blotting detection reagents (GE Healthcare, Chalfont, UK) and visualized using a FUSION Solo Chemiluminescence System (PEQLAB Biotechnologie GmbH, Germany).

Statistical analysis. Differences between treatments were evaluated by one-way analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

The MeOH extract of *M. alba* fruits was partitioned with hexane, CHCl₃, EtOAc, and n-BuOH. The CHCl₃-soluble fraction was subjected to a series of open-column chromatography and Sephadex-LH20 column chromatography, and then purified by semi-preparative HPLC, to obtain six heterocyclic compounds (1-6) (Fig. 1). The chemical structures of the isolated compounds were unambiguously elucidated to be (R)-5-hydroxypyrrolidin-2-one (1, 15), methyl (R)-pyroglutamate (2, 16), loliolide (3, 17), 3-benzofuran-carboxaldehyde (4, 18), odisolane (5, 19), and indole (6, 20) by comparing their NMR spectroscopic values and physical properties with previously reported literature data.

We first examined the effects of all compounds on the cell viability decreased by STZ in INS-1 cells. Cells were pre-treated with the indicated concentrations of compounds 1-6 (0 to 100 µM of each compound) for 2 h and further exposed to 50 µM STZ for 24 h. As shown in Fig. 2, the exposure to 50 µM STZ for 24 h decreased cell viability (58.62±0.17%) compared to control treatment (100%). Among the six compounds, compound 3 showed the strongest protective effect on STZ-induced INS-1 cytotoxicity in a concentration-dependent manner, whereas the other compounds showed minimal or no effect (Fig 2A-F). Compound 3 showed maximum effect at the concentration of 100 µM, as indicated by increased cell viability (80.27±3.48%).

The increase in the levels of intracellular ROS is a key characteristic in STZ-induced pancreatic β-cell death (21). Therefore, we then examined the antioxidative effects of compounds 1-6. Consistent with previous studies, we found, as shown in Fig. 3, a remarkable increase in intracellular ROS after...
exposure to STZ for 24 h. In contrast, compounds 3 (Fig. 3C) and 5 (Fig. 3E) significantly reduced the levels of intracellular ROS that were increased by STZ in INS-1 cells, whereas the other compounds were not effective (Fig. 3A, B, D, F). Based
on the results of both the cell viability assay and the ROS measurement studies, we found that compound 3 was the most effective in preventing INS-1 cell death and the production of ROS induced by STZ. Therefore, we further investigated not only the anti-apoptotic effect of compound 3 but also its underlying mechanism against STZ-induced apoptotic cell death.

It has been reported that STZ induces apoptosis and necrosis at low and high concentrations respectively and that both apoptosis and necrosis contribute to the development of type 1 diabetes (22,23). Therefore, we examined the effect of compound 3 against STZ-induced apoptosis in INS-1 cells. The morphological images in Fig. 4A show that the presence of compound 3 strongly prevented STZ-induced apoptosis in INS-1 cells (Fig. 4A). To determine the anti-apoptotic effect, cells were stained with Annexin-V Allexa 488 after exposure to 50 µM STZ in the presence of 50 and 100 µM compound 3. The representative photographs show that the treatment with compound 3 markedly reduced the Annexin V-positive cells (Fig. 4B). In addition to this, we quantitatively analyzed apoptotic cells by the percentage of Annexin V-positive cells relative to total cells. As shown in Fig. 4C, the percentage of apoptotic cells dramatically increased by the exposure to STZ (14.66±1.15%) while it was significantly reduced in the presence of 50 (9.66±1.52%) and 100 µM (4.33±1.15%) compound 3 (Fig. 4C).

STZ induces apoptosis via activation of caspase-8 and caspase-3 and regulation of the protein expression of Bcl-2 family members in INS-1 cells (24). Moreover, we recently reported that the inhibition of caspase-8 and caspase-3 by cirsimaritin prevented apoptosis in INS-1 cells as well as increased Bcl-2 protein expression (25). This suggests that the inhibition of the caspase signaling pathway is a possible target to protect pancreatic cell death in type 1 diabetes. We further investigated to determine the underlying protective mechanism of compound 3 against STZ-induced apoptotic INS-1 cell death using western blot analysis for pro-apoptotic and anti-apoptotic proteins. As shown in Fig. 5A, cleavage of caspase-8, caspase-3, and PARP was markedly increased after treatment with 50 µM STZ, whereas it decreased in the presence of 50 and 100 µM of compound 3 in a concentration-dependent manner (Fig. 5B). However, the ratio of Bax to Bcl-2 indicating

Figure 5. Underlying protective mechanism of compound 3 against STZ-induced apoptotic cell death. (A) Cells were exposed to 50 µM STZ for 24 h after pretreatment with 50 and 100 µM of compound 3 for 2 h. (B) The graph represents the fold increase in cleaved caspase-8, cleaved caspase-9, and PARP. GAPDH was used as a loading control. Treatment with compound 3 decreased the cleavage of caspase-8, caspase-3, and PARP and increased Bcl-2 protein expression. (C) The mechanism of protection mediated by compound 3 against STZ-induced INS-1 cell death. *P<0.05 compared with STZ only treated cells. STZ, streptozotocin; ROS, reactive oxygen species; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP, poly (ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2.
mitochondrial apoptotic pathway was altered neither cisplatin only-nor cisplatin with compound 3-treated cells (Fig 5A and B). This result indicated that compound 3 exhibits anti-apoptotic activity via blocking the activation of caspase-8 and caspase-3 and inducing PARP cleavage (Fig. 5C).

In the present study, we found that compound 3 prevented STZ-induced apoptotic pancreatic β cell death via the inhibition of the caspase signaling pathway and induction of Bcl-2 protein expression. Therefore, this study suggests that compound 3, a strong bioactive natural compound from the extracts of *M. alba*, may be a suitable therapeutic for type 1 diabetes.

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