

## Effects of novel chalcone derivatives on $\alpha$ -glucosidase, dipeptidyl peptidase-4, and adipocyte differentiation *in vitro*

Eun Jung Bak<sup>1</sup>, Hong Gyu Park<sup>2</sup>, ChoongHwan Lee<sup>3</sup>, Tong-Il Lee<sup>3</sup>, Gye-Hyeong Woo<sup>4</sup>, Younghwa Na<sup>7</sup>, Yun-Jung Yoo<sup>5,6,\*</sup> & Jeong-Heon Cha<sup>1,5,6,\*</sup>

<sup>1</sup>Oral Cancer Research Institute, Yonsei University College of Dentistry, Seoul 120-749, <sup>2</sup>Atgc Biotechnology Co., Ltd, Seongnam 461-713, <sup>3</sup>ATGen Co., Ltd, Seongnam 463-070, <sup>4</sup>Department of Clinical Laboratory Science, Semyung University, Jecheon 390-711, <sup>5</sup>Department of Oral Biology, BK21 Project, Oral Science Research Center, Research Center for Orofacial Hard Tissue Regeneration, Yonsei University College of Dentistry, <sup>6</sup>Department of Applied Life Science, The Graduate School, Yonsei University, Seoul 120-749, <sup>7</sup>College of Pharmacy, CHA University, Pocheon 487-801, Korea

**Chana series are new chalcone derivatives. To evaluate the possibility of Chana series as therapeutic agents of type 2 diabetes, the inhibitory effects of Chana series on the activities of  $\alpha$ -glucosidase and DPP-4 were investigated using *in vitro* enzyme assays, and their effects on adipocyte differentiation were investigated in C3H10T1/2 cells. Chana 1 and Chana 7 among the Chana series showed significant inhibition of  $\alpha$ -glucosidase activity. In DPP-4 enzyme assay, Chana 1 exhibited the highest inhibitory activity while Chana 7 did not. In MTT assay, Chana 1 did not show significant cytotoxicity up to a concentration of 250  $\mu$ M, whereas cytotoxicity was observed with Chana 7 at a concentration of 300  $\mu$ M. In addition, Chana 1 induced adipocyte differentiation. Therefore, Chana 1 showed inhibitory effects on  $\alpha$ -glucosidase and DPP-4 as well as a stimulatory effect on adipocyte differentiation, suggesting that Chana 1 may be a potential beneficial agent for the treatment of type 2 diabetes. [BMB reports 2011; 44(6): 410-414]**

### INTRODUCTION

Diabetes has become increasingly common around worldwide. Type 2 diabetes, in particular, is caused by a metabolic disorder characterized by hyperglycemia and insulin resistance (1-3). Various therapeutic approaches have been used to alleviate diabetes, including improvement of insulin sensitivity, inhibition of gluconeogenesis, and reduction of absorption of glucose from the intestine (4). The goal of treatment of type 2 diabetes mellitus is generally maintenance of glycemic control

in the postprandial state as well as the fasting state (5).

$\alpha$ -Glucosidase is a membrane-bound enzyme at the epithelium of the small intestine and plays a key role in carbohydrate digestion (6). Inhibition of  $\alpha$ -glucosidase leads to the delay or reduction of increased postprandial blood glucose levels. Acarbose, a well established  $\alpha$ -glucosidase inhibitor, significantly decreases postprandial plasma glucose and HbA1c levels (7). Thus,  $\alpha$ -glucosidase inhibitors have been proposed as a potential therapeutic target for drug discovery in the treatment of type 2 diabetes (8, 9).

Two gut hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), function as incretins that increase insulin secretion from  $\beta$ -cells after enteral nutrient ingestion (10). Recently, clinical evidence has suggested that incretin-based therapies may be useful for the management of type 2 diabetes (11, 12). Dipeptidyl peptidase-4 (DPP-4) inhibitors improve hyperglycemic conditions by stabilizing postprandial levels of GLP-1 and GIP in patients with type 2 diabetes and have been approved as a target for the treatment of type 2 diabetes (13, 14).

Licorice, which is used as a traditional medicine, originates from the root and stolon of *Glycyrrhiza* plant species (Leguminosae). Six retrochalcone, licochalcone A-E, and echinatin have been isolated from the roots of *G. inflata*, a *Glycyrrhiza* species member. It was previously reported that these retrochalcones have various biological activities, including anti-cancer, anti-bacterial, anti-oxidative, and anti-inflammatory effects (15-17). In this study, we synthesized new chalcone derivatives, Chana series, and evaluated their novel inhibitory effects on the enzyme activities of  $\alpha$ -glucosidase and DPP-4 and adipocyte differentiation *in vitro*.

### RESULTS AND DISCUSSION

#### Effects on $\alpha$ -glucosidase activity

To identify  $\alpha$ -glucosidase inhibitors, 10 pure synthetic compounds of new retrochalcone derivatives were used in this assay. *In vitro*  $\alpha$ -glucosidase assay found that both Chana 7

\*Corresponding author. Yun-Jung Yoo, Tel:82-2-2228-3060; Fax: 82-2-2227-7903; E-mail: yu618@yuhs.ac; Jeong-Heon Cha, Tel:82-2-2228-3061; Fax: 82-2-2227-7903; E-mail: Jcha@yuhs.ac  
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and Chana 1 had more potent effects than other Chana series (Table 1). The inhibition rates of Chana 7 and Chana 1 at a concentration of 1 mM were 92.1% and 63.1%, respectively. Thus, we selected Chana 7 due to its high inhibition rate and examined its  $\alpha$ -glucosidase inhibition activity at various concentrations (0.062-1 mM). Chana 7 inhibited  $\alpha$ -glucosidase activity in a concentration-dependent manner (Fig. 1). Interestingly, Chana 7 inhibited  $\alpha$ -glucosidase more effectively than acarbose, the positive control. Therefore, Chana 1 and Chana 7 showed strong inhibitory effects on  $\alpha$ -glucosidase activity. By extension, these results suggest that Chana 1 and Chana 7 are effective  $\alpha$ -glucosidase inhibitors.

#### Effects on DPP-4 activity

The effects of Chana 1 and Chana 7 on DPP-4 activity were measured using *in vitro* enzyme assay. As shown in Table 2, Chana 1 at a concentration of 1 mM significantly decreased DPP-4 activity to 48.1%. However, Chana 7 at a concentration of 1 mM did not show any effect on DPP-4 activity. Therefore, Chana 1 inhibited DPP-4 activity, which implies it could be a novel therapeutic agent for the treatment of type 2 diabetes.

#### Effects on cell viability

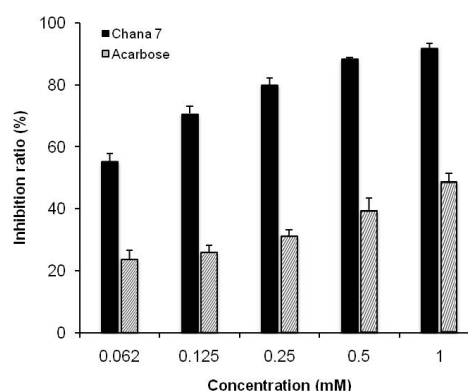
MTT assay was conducted after treatment with Chana 1 and Chana 7 for 48 h at concentrations of 50-1,200  $\mu$ M. The results demonstrate that Chana 1 exerted no inhibitory effects on cell viability up to a concentration of 250  $\mu$ M (Fig. 2A), whereas cell viability in the presence of Chana 7 was significantly reduced at a concentration of 300  $\mu$ M (Fig. 2B). Therefore, the side effect of Chana 1 on cell viability was lower than that of Chana 7.

#### Effect on adipocyte differentiation of C3H10T1/2 multipotent stem cells

To determine the effects of Chana 1 on adipocyte differentiation, C3H10T1/2 multipotent stem cells were treated with Chana 1 at a concentration of 10  $\mu$ M for 8 days during stand-

ard adipogenic induction. Rosiglitazone (rosi), a PPAR  $\gamma$  agonist, was used as a positive control of adipocyte differentiation. The rosi positive control strongly induced adipocyte differentiation in C3H10T1/2 cells (Fig. 3A). Treatment with Chana 1 at a concentration of 10  $\mu$ M induced lipid accumulation, as detected by both Oil red O staining for lipid droplets and spectrophotometric quantification of extracted Oil red O stains (Fig. 3A and B). Therefore, Chana 1 induced adipocyte differentiation. In previous studies, *in vitro* adipocyte differentiation as a model for testing insulin sensitivity was used to screen novel anti-diabetic drugs (18, 19). Further, several studies reported that natural pure compounds that promote adipocyte differentiation can be used for the treatment of type 2 diabetes (20, 21). Therefore, Chana 1 may have exerted a beneficial effect on type 2 diabetes.

In conclusion, Chana 1 and Chana 7 demonstrated inhibitory activity on  $\alpha$ -glucosidase, and Chana 1 had an inhibitory effect on DPP-4. In addition, Chana 1 stimulated adipocyte differentiation *in vitro*. These results may provide evidence that a new chalcone derivative, Chana 1, is a novel agent for the treatment of type 2 diabetes. Further study is needed to prove whether or not chalcone derivatives improve diabetes *in vivo*.



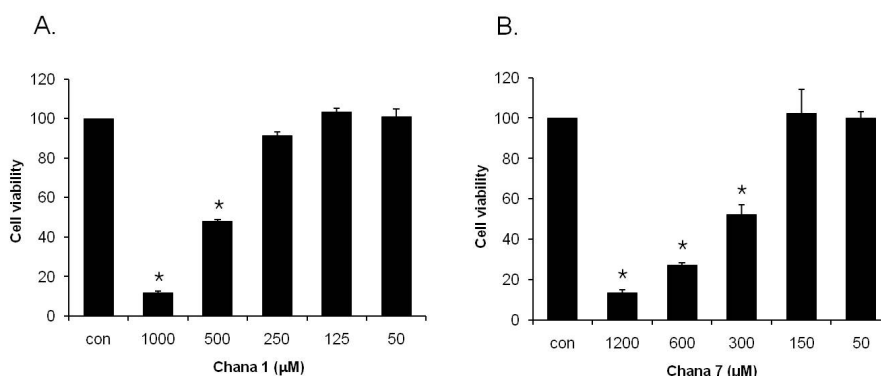
**Fig. 1.** Effect of Chana 7 on  $\alpha$ -glucosidase activity *in vitro*.  $\alpha$ -Glucosidase was dissolved at a concentration of 2 units/ml and was used as an enzyme source; 5 mM *para*-Nitrophenyl- $\alpha$ -D-glucopyranoside was used as a substrate. Ten microliters of each Chana 7 dilution or Acarbose were mixed with 50  $\mu$ l of reaction buffer and 20  $\mu$ l of enzyme. Finally, 20  $\mu$ l of substrate was added and incubated for 10 min at 37°C. After incubation, the absorbance was measured at 405 nm. The error bars represent the standard error of the mean.

**Table 1.** Effects on  $\alpha$ -glucosidase inhibition activity of synthesized Chana series

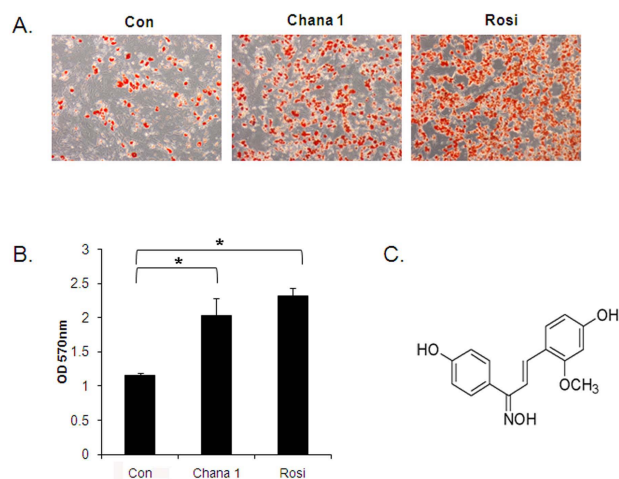
Compound (1 mM)	$\alpha$ -glucosidase
	Inhibition ratio (%)
Chana 1	63.1 $\pm$ 3.3
Chana 2	13 $\pm$ 1.5
Chana 3	32.1 $\pm$ 2.8
Chana 4	9.2 $\pm$ 0.9
Chana 5	1.8 $\pm$ 1.1
Chana 6	18.9 $\pm$ 1.7
Chana 7	92.1 $\pm$ 2.2
Chana 8	22.5 $\pm$ 2.0
Chana 9	19.4 $\pm$ 1.2
Chana 10	27.3 $\pm$ 2.2

**Table 2.** DPP-4 inhibitory activity of Chana 1 and Chana 7

Compound (1 mM)	DPP-4
	Inhibition ratio (%)
Chana 1	48.1 $\pm$ 2.4
Chana 7	0



**Fig. 2.** Effects of Chana 1 and Chana 7 on cell viability. (A) To investigate the toxicity of Chana 1 on cell viability, PC 3 cells were incubated in the presence of Chana 1 at concentrations of 0, 50, 125, 250, 500, and 1,000 μM, after which cell viability was evaluated via MTT assay after 48 h. (B) To investigate the toxicity of Chana 7 on cell viability, PC 3 cells were incubated in the presence of Chana 7 at concentrations of 0, 50, 150, 300, 600, and 1,200 μM, after which was evaluated via MTT assay after 48 h. The error bars represent the standard error of the mean.



**Fig. 3.** Effect of Chana 1 on adipocyte differentiation of C3H10T1/2 cells. (A) C3H10T1/2 cells were treated with Chana 7 (10 μM) or rosiglitazone (Rosi, 1 μM) during adipocyte differentiation for 8 days. Control was differentiated by standard adipogenic induction media without treatment. The adipogenesis of C3H10T1/2 cells was visualized by Oil Red O staining. Microscopic pictures were taken at day 8 with ×100 magnification. (B) Oil Red O stain was extracted with isopropanol. The absorbance of the extracted Oil Red O stain was determined spectrophotometrically at 570 nm in order to measure triglyceride (TG) accumulation. The symbol \* indicates significant difference at  $P < 0.01$ . (C) The chemical structure of Chana 1.

## MATERIALS AND METHODS

### α-Glucosidase enzyme assay

Inhibitory activity against α-glucosidase was measured according to the method described previously (8) with minor modi-

fications. Briefly, α-glucosidase (Sigma, St Louis, MO, USA) was dissolved at a concentration of 2 units/ml in reaction buffer (100 mM phosphate buffer and 0.02% sodium azide) and used as an enzyme source; 5 mM para-Nitrophenyl-α-D-glucopyranoside (Sigma, St Louis, MO, USA) was used as a substrate. Firstly, we carried out the α-glucosidase inhibition assay with Chana series at a concentration of 1 mM. Then, solutions of Chana 7 among the Chana series at concentrations of 0.062, 0.125, 0.25, 0.5, and 1 mM were made by serial dilution. Ten microliters of each Chana 7 dilution was mixed with 50 μl of reaction buffer and 20 μl of enzyme source. Finally, 20 μl of substrate was added and incubated for 10 min at 37°C. After incubation, the O.D. was measured at 405 nm using an MRX II microplate reader (Dynatech Labs., Chantilly, VA, USA). Acarbose was used as a positive control. The α-glucosidase inhibition ratio was calculated as follows: α-glucosidase inhibition ratio (%) = [(O.D. of control × O.D. of test sample) / O.D. of control] × 100. All assays were conducted in triplicate, and at least three separate assays were performed.

### DPP-4 enzyme assay

Inhibitory activity against DPP-4 was measured by the method proposed by Tanaka-Amino et al. (22) with minor modifications. Briefly, DPP-4 activity was determined by the rate of hydrolysis of a surrogate, H-Gly-Pro-AMC (AMC; 7-amino-4-methylcoumarine). Then, 20 μl of enzyme was added to each well of a black 96-well flat-bottom plate, followed by addition of 20 μl of assay buffer (25 mM HEPES, 140 mM NaCl, 1% RIA-grade BSA, pH 7.8) containing 80 mM MgCl<sub>2</sub>, followed by 20 μl of Chana 1 and 7 at the indicated concentrations. The reaction was initiated by adding 20 μl of assay buffer containing 200 μM H-Gly-Pro-AMC. After being incubated at room temperature for 15 min, the fluorescence emitted by liberated AMC was measured at 405 nm using an MRX II microplate reader

(Dynatech Labs., Chantilly, VA, USA). All assays were conducted in triplicate, and at least three separate assays were performed

### 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay

The cells ( $1 \times 10^4$  cells/well) were plated and incubated for 24 h in 96-well plates with the indicated concentrations of Chana 1 and Chana 7. After 48 h, MTT (Sigma-Aldrich, St. Louis, MO, USA) was employed to measure cell viability, in accordance with the manufacturer's instructions. The absorbance at 570 nm was measured with an MRX II microplate reader (Dynatech Labs, Chantilly, VA, USA).

### Adipocyte differentiation assay

C3H10T1/2 cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin in a humidified incubator under an atmosphere of 5% CO<sub>2</sub> at 37°C. C3H10T1/2 cells at 2 days post-confluence (day 0) were stimulated to differentiate in standard adipogenic medium (DMEM containing 10% FBS, 0.5 µM isobutylmethylxanthine, 1 µM dexamethasone, and 1 µg/ml of insulin) for 2 days (23, 24). The differentiation medium was replaced after 2 days with DMEM containing 10% FBS and 1 µg/ml of insulin, followed by exchange with DMEM containing 10% FBS every day thereafter. During adipocyte differentiation, C3H10T1/2 cells were treated with 10 µM Chana 1 from days 0 to 8, followed by treatment with 1 µM rosi as a positive control. All assays were conducted in triplicate, and at least three separate assays were performed.

### Quantification of lipid accumulation

In order to determine the degree of differentiation and visualize lipid accumulation, cytoplasmic triglycerides in the cells were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, US) as previously described (23, 24). The stained cells were photographed at a magnification of  $\times 100$  using an Olympus CKX41 inverted microscope system (Tokyo, Japan). After the Oil Red O retained in the cells was extracted with isopropanol, the absorbance was determined spectrophotometrically at 570 nm using an MRX II microplate reader (Dynatech Labs., Chantilly, VA, USA).

### Statistical analysis

The SPSS 12.0 statistical package program (SPSS Inc. Chicago, IL, USA) was utilized for all statistical analyses. All data in the same groups were assessed via paired t-tests. A P value of  $< 0.05$  was considered to be indicative of a statistically significant difference.

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