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# Effects of a new sustained-release microsphere formulation of exenatide, DA-3091, on obese and non-alcoholic fatty liver disease mice

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The aim of this study was to examine the effects of a new sustained-release (SR) microsphere formulation of exenatide, DA-3091, on body weight gain and hepatic injury in high fat diet (HFD)-induced obese mice and high sucrose diet (HSD)-induced non-alcoholic fatty liver disease (NAFLD) mice. Then, we determined whether DA-3091 has the potency as a drug for the treatment of metabolic disease. In obese mice, after 8-week treatment, the body weight gain was significantly more suppressed by both 1 mg/kg and 2 mg/kg of DA-3091, monthly subcutaneous administered, than by 10 mg/kg/day of sibutramin, a drug against obesity. In NAFLD mice, a significant reduction in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, representative markers of hepatic injury, was observed after biweekly subcutaneous administration of 1 mg/kg and 2 mg/kg of DA-3091 for 8 weeks. A significant reduction in hepatic lipid accumulation was observed in DA-3091 treated groups as well. Based on these results, it is demonstrated that DA-3091 has the potency as a drug for the treatment of metabolic disease.

## 1. Introduction

Obesity is a risk factor for the development of diabetes, hyperglycemia, hyperinsulinemia, insulin resistance, and dyslipidemia (Maggio and Pi-Sunyer 2003) and also for non-alcoholic fatty liver disease (NAFLD), hypertension, and cardiovascular disease (Mokdad et al. 2003; Bray 2007). Recent basic and epidemiologic data reveal that NAFLD is a hepatic manifestation of metabolic syndrome that is closely associated with multiple factors such as obesity, hypertension as well as a constellation of clinical problems that arise from insulin resistance (Abdelmalek and Diehl 2007). Moreover, NAFLD is an increasingly common health concern that is considered to be a component of the metabolic syndrome (Postic and Cirard 2008) and tightly associated with obesity and insulin resistance (Dowman et al. 2011). To date, no effective monotherapy for the prevention or treatment of NAFLD has been developed. The complicated pathogenesis of NAFLD suggests that combination therapy may be more effective for the treatment of NAFLD (Nozaki et al. 2009).

The incretin mimetic exenatide, the first glucagon like-peptide-1 (GLP-1) receptor agonist to be approved for therapeutic use in humans, is a 39-amino acid peptide originally isolated from the salivary secretions of *Heloderma suspectum* (Gila monster) and it shares approximately 53% sequence homology with the mammalian gut hormone, GLP-1 (En[1]g et al. 1992). Consequently, exenatide shares many glucoregulatory actions with GLP-1 including glucose-dependent insulinotropic, glucagonostatic, gastric slowing and satiogenic effects *in vitro* (Nielsen and Baron 2003). However, exenatide has a short half-life in circulation, and needs to be administered by twice-daily subcutaneous injection (Buse et al. 2004). Hence, we already developed a new sustained-release microsphere formulation of exenatide, DA-3091, using PLGA (poly(lactic-co-glycolic acid)) by oil-in-water (o/w) emulsion solvent evaporation method (Kwak et al. 2009). Exenatide from DA-3091 was sustainably released during 2 to 6 weeks after a single subcutaneous injection to mice, rats and monkeys. The anti-diabetic effects of DA-3091 after a single subcutaneous injection per 1 to 4 week were shown to be dose dependent (0.1 ∼ 2 mg/kg) in Zucker diabetic fatty rats [ZDF/Gmi-(fa/fa)] (Kwak et al. 2010).

The purpose of this study was to additionally examine the prolonged effects of DA-3091 on body weight gain and hepatic injury in obese mice and NAFLD mice which were induced by high fat diet and high sucrose diet, respectively. Then, we determined whether DA-3091 has the potency as a drug for the treatment of metabolic disease.

## 2. Investigations and results

### *2.1. Effects of DA-3091 on high fat diet (HFD)-induced obese mice*

Obesity in mice was induced by feeding HFD for 4 weeks. Then, DA-3091 (1 or 2 mg/kg) and sibutramin (10 mg/kg) were monthly subcutaneously administrated and daily orally administered, respectively, to HFD-fed mice for the following 8 weeks. DA-3091 treatment suppressed the body weight gain induced by HFD-fed. After the first injection, 1 mg/kg and 2 mg/kg of DA-3091 significantly suppressed body weight gain for 2 weeks and 4 weeks, respectively, and after the second injection, both 1 mg/kg and 2 mg/kg of DA-3091 significantly suppressed body weight gain for 4 weeks, compared

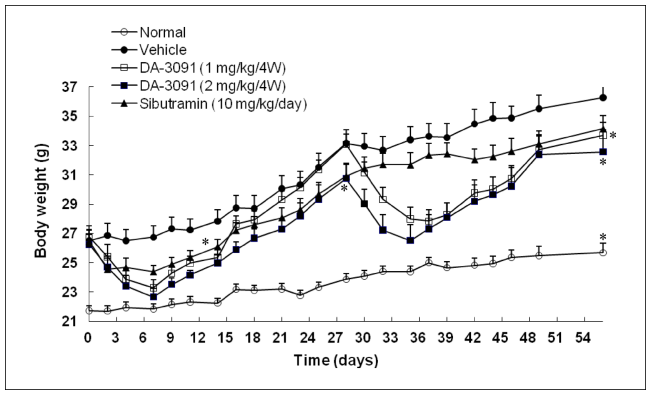



Fig. 1: Effects of monthly subcutaneous injection of 1 mg/kg and 2 mg/kg of DA-3091 on the body weight compared to daily oral administration of 10 mg/kg sibutramin in HFD-induced obese mice. Data are expressed as a mean ± SEM. *$P < 0.05$ vs. vehicle treated group

to vehicle treated group (6.9 ± 0.7 g, 6.3 ± 1.3 g vs. 9.6 ± 0.7 g, respectively) (Fig. 1). However, sibutramin treatment showed no significant difference in body weight gain compared to vehicle treated group (7.9 ± 0.9 g vs. 9.6 ± 0.7 g). And also, DA-3091 treatment reduced food intake compared to vehicle and sibutramin treated groups (Fig. 2).

### *2.2. Effects of DA-3091 on high sucrose diet (HSD)-induced non-alcoholic fatty liver disease (NAFLD) mice*

#### *2.2.1. Body weight and liver mass*

In NAFLD mice, increased body weight and liver mass were induced by feeding HSD for 6 weeks and then, DA-3091 (1 or 2 mg/kg) was biweekly administered subcutaneously to HFD-fed mice for the following 8 weeks. The absolute body weight of each group was measured at the start and the end of DA-3091 treatment period and the body weight gain was calculated as presented in the Table. The body weight gain was significantly suppressed in both 1 mg/kg and 2 mg/kg of DA-3091 treated groups, compared to vehicle treated group (3.3 ± 0.9 g, 1.3 ± 0.8 g vs. 8.1 ± 0.9 g, respectively). Although 1 mg/kg and 2 mg/kg of DA-3091 administered groups showed a dose dependent pattern in reduction of body weight gain, there was no statistically significant difference. The liver weight was 1.00 ± 0.05 g and 1.03 ± 0.02 g in both 1 mg/kg and 2 mg/kg of DA-3091 treated groups, which was significantly lower compared to vehicle treated group's (1.39 ± 0.09 g) and similar to that of normal group's (1.00 ± 0.02 g) (Table). However, the liver weight to total body weight ratio (%) was not significantly different.

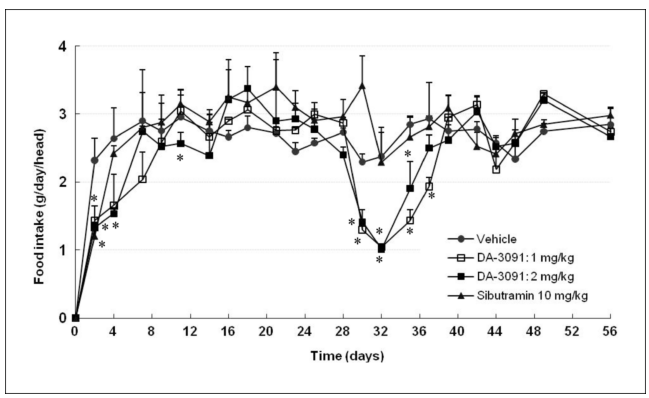
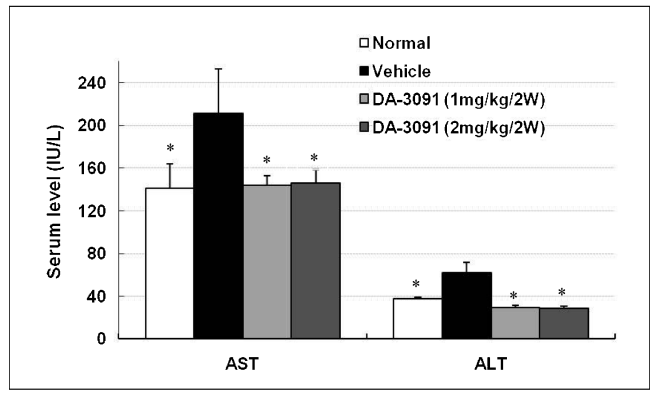
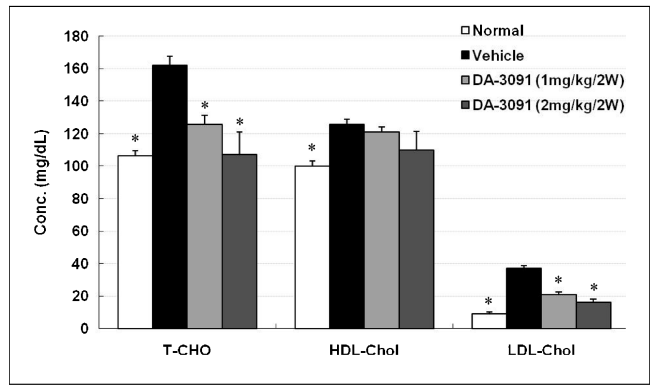



Fig. 2: Effects of monthly subcutaneous injection of 1 mg/kg and 2 mg/kg of DA-3091 on the daily food intake compared to daily oral administration of 10 mg/kg sibutramin in HFD-induced obese mice. Data are expressed as a mean ± SEM. *$P < 0.05$ vs. vehicle treated group




(A)




(B)

Fig. 3: Effects of the biweekly subcutaneous injection of 1 mg/kg and 2 mg/kg of DA-3091 on AST and ALT level (A) and serum cholesterol level (B) in HSD-induced NAFLD mice. Data are expressed as a mean ± SEM. *$P < 0.05$ vs. vehicle treated group

#### *2.2.2. Measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cholesterol levels*

At the end of the experiment, the levels of representative markers of hepatic injury like serum ALT, AST and cholesterol were measured. Serum ALT and AST levels were significantly increased about 1.7- and 1.5-fold in HSD-fed mice, respectively. However, the serum ALT and AST levels in 1 mg/kg and 2 mg/kg of DA-3091 treated groups were significantly lowered compared to the vehicle treated group, which showed similar levels than the normal group (Fig. 3A). And also, DA-3091 treatment suppressed the increased serum total cholesterol level (1.5-fold) induced by HSD-fed. The total cholesterol level was markedly ($p < 0.05$) lower in the DA-3091 treated group (1 mg/kg: 125.6 ± 5.8 mg/dL, 2 mg/kg: 107.0 ± 14.0 mg/dL) than in the vehicle treated group (162.0 ± 5.5 mg/dL). Moreover, the low-density lipoprotein (LDL)-cholesterol levels were significantly ($p < 0.05$) lower in the DA-3091 treated group (1 mg/kg: 21.1 ± 1.6 mg/dL, 2 mg/kg: 16.2 ± 1.9 mg/dL) than in the vehicle treated group (37.2 ± 1.4 mg/dL). Among the high-density lipoprotein (HDL)-cholesterol levels, there was no significant difference (Fig. 3B).

#### *2.2.3. Liver histological evaluation*

A histological examination showed that the liver from the standard diet fed animals was normal, whereas a hepatic fat infiltration was observed in the HSD-fed group without inflammation or obvious hepatic cell fibrosis. Figure 4 shows that 1 mg/kg and 2 mg/kg of DA-3091 treatment significantly reduced hepatic lipid accumulation induced by HSD-fed, which was confirmed by hematoxylin and eosin (HE) staining.

**Table: Effects of DA-3091 on the body weight and liver mass in HSD-induced NAFLD mice**

| Groups | Initial body weight (g) | Final body weight (g) | Δ-body weight (g) | Liver mass (g) | Liver to body weight (%) |
|---|---|---|---|---|---|
| Normal | 21.5 ± 0.4 | 25.9 ± 0.5 | 4.4 ± 0.3 | 1.00 ± 0.02 | 3.9 ± 0.0 |
| Vehicle | 24.2 ± 0.6 | 32.3 ± 0.9 | 8.1 ± 0.9 | 1.39 ± 0.09 | 4.3 ± 0.2 |
| DA-3091 (1 mg/kg, biweekly) | 23.9 ± 0.5 | 27.2 ± 0.8 * | 3.3 ± 0.9 * | 1.00 ± 0.05 * | 3.9 ± 0.2 |
| DA-3091 (2 mg/kg, biweekly) | 24.4 ± 0.3 | 25.7 ± 0.7 * | 1.3 ± 0.8 * | 1.03 ± 0.02 * | 4.0 ± 0.1 |

Data are expressed as mean ± SEM. Δ-body weight means change in body weight from initial body weight to final body weight. * $P < 0.05$ vs. vehicle treated group.

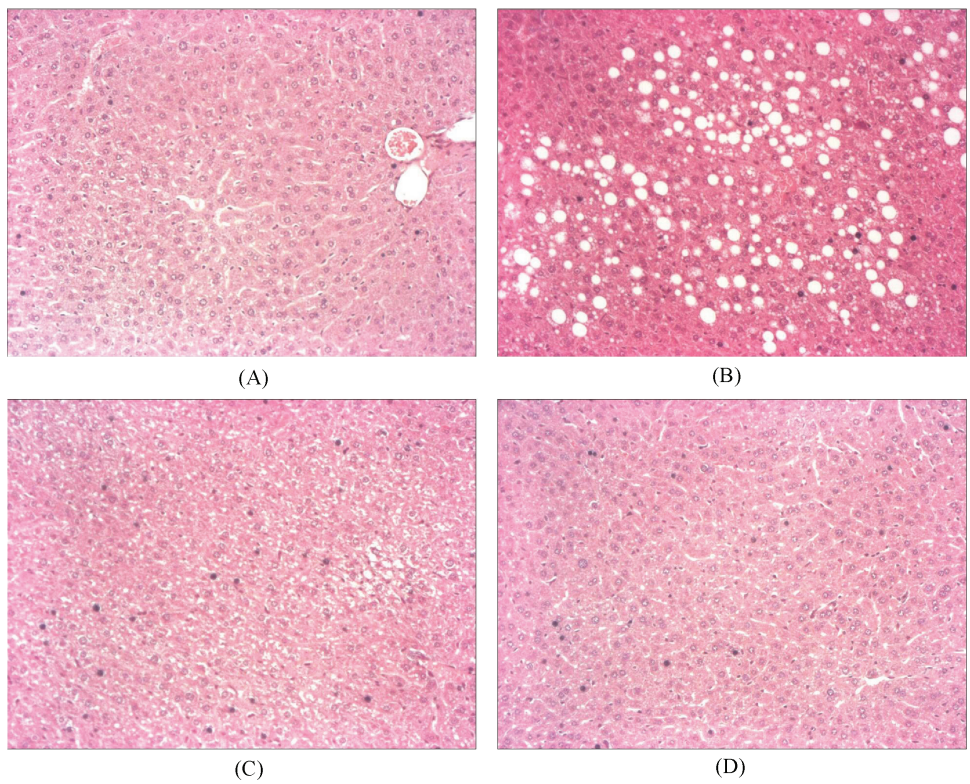



Fig. 4: Effects of the biweekly subcutaneous injection of DA-3091 on the fatty liver in HSD-induced NAFLD mice by the liver tissues HE-staining. (A) is standard diet fed group, (B) is HSD-fed and vehicle treated group, (C) is HSD-fed and 1 mg/kg of DA-3091 treated group, and (D) is HSD-fed and 2 mg/kg of DA-3091 treated group

### *2.2.4. Serum glucose level and endotoxin level*

HSD-fed increase levels of glucose and endotoxin in serum (Spruss et al. 2009). Actually, in this study, HSD-feeding for 14 weeks in total significantly increased fasting serum glucose and endotoxin levels by 3.2- and 4.8-fold, respectively, compared to a standard diet fed group. At the starting of DA-3091 treatment, the fasting serum glucose levels were similar in all experimental groups. But after 8-week DA-3091 treatment, serum glucose level was significantly lowered in 1 mg/kg and 2 mg/kg of DA-3091 treated groups compared to the vehicle treated group (97 ± 8 mg/dL, 97 ± 4 mg/dL vs. 232 ± 20 mg/dL, respectively, $p < 0.05$), with levels similar to that of the normal group (Fig. 5). As shown in Fig. 6, DA-3091 treatment also significantly decreased the serum endotoxin levels compared to the vehicle treated group (17.1 ± 4.8 EU/mL and 17.4 ± 3.5 EU/mL vs. 44.7 ± 15.4 EU/mL, respectively, $p < 0.05$).

## 3. Discussion

The metabolic syndrome, a cluster of cardiovascular disease factors, is present in the majority of obese type 2 diabetic patients (Dekker et al. 2005). Fat accumulation in the liver or NAFLD is regarded as a key pathogenic factor and component of this syndrome (Den Boer et al. 2004). Although the pathogenesis of NAFLD remains poorly understood, NAFLD is one of the most reproducible factors in the development of metabolic syndrome (Expert Panel 2001). The five components that compose the metabolic syndrome are central (truncal) obesity, hyperglycemia, hypertension, hypertriglyceridemia, and low levels of HDL-Cholesterol. Subjects with specified values for at least three of these components are considered to have metabolic syndrome.

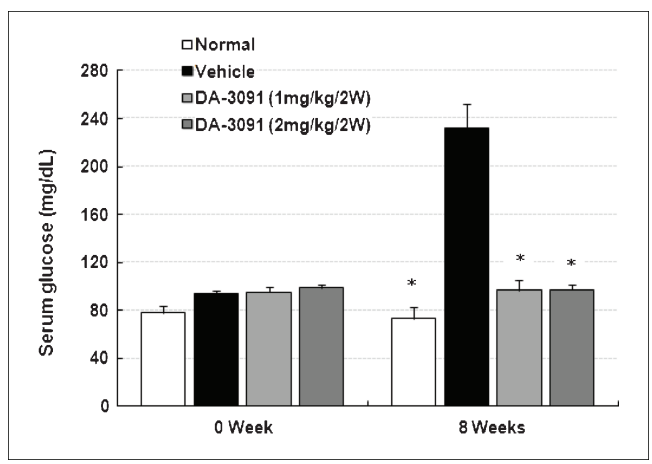



Fig. 5: Effects of the biweekly subcutaneous injection of 1 or 2 mg/kg of DA-3091 on the serum glucose level in HSD-induced NAFLD mice. Data are expressed as a mean ± SEM. * $P < 0.05$ vs. vehicle treated group

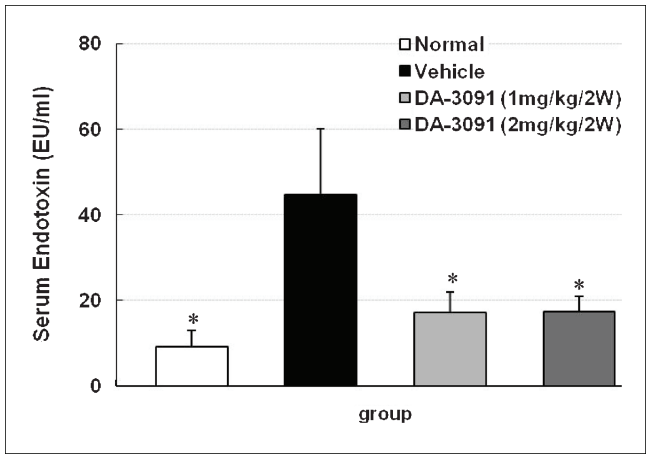



Fig. 6: Effects of the biweekly subcutaneous injection of 1 or 2 mg/kg of DA-3091 on the serum endotoxin level in HSD-induced NFALD mice. Data are expressed as a mean ± SEM. *$P < 0.05$ vs. vehicle treated group

The incretin mimetic exenatide, which shares many glucoregulatory actions with GLP-1 including glucose-dependent insulinotropic, glucagonostatic, gastric slowing and satiogenic effects (Nielsen et al. 2004) for the treatment of metabolic syndrome, but exenatide has a short half-life in circulation, and needs to be administered by twice-daily subcutaneous injection (Buse et al. 2004). Hence, we developed a new sustained-release microsphere formulation of exenatide, DA-3091, releasing sustainably exenatide during 2 to 6 weeks after a single subcutaneous injection to mice, rats and monkeys (Kwak et al. 2009). The period of exenatide release was increased in a dose-dependent manner according to the injected DA-3091 doses. The anti-diabetic effects of DA-3091 after a single subcutaneous injection per 1- to 4-week were exerted in a dose-dependent manner (0.1∼2 mg/kg) in Zucker diabetic fatty rats [ZDF/Gmi-(fa/fa)] (Kwak et al. 2010).

In addition to the previous results, we assessed the long acting effects of DA-3091 on body weight gain and hepatic injury in obese mice and NAFLD mice. In obese mice, DA-3091 treatment significantly suppressed the body weight gain and reduced food intake. These results suggest that DA-3091 has anorectic effects in obese mice, consistent with the results of previous studies. Exenatide has anorectic effects by its action on the hypothalamus to increase satiety and slow gastric emptying (Kim and Egan 2008; Drucker 2003). After the first injection, 1 mg/kg of DA-3091 significantly suppressed the body weight gain for 2 weeks only, but 2 mg/kg of DA-3091 significantly suppressed it for 4 weeks. And, after the second injection, both doses significantly suppressed the body weight gain for 4 weeks. Considering the exenatide releasing profile of DA-3091, which releases exenatide over minimum effective concentration in a dose-dependent manner, we expect that, in single-dose regimen, 1 mg/kg of DA-3091 is a proper dose for biweekly treatment to suppress body weight gain and 2 mg/kg of DA-3091 is a proper dose for monthly treatment. Though, in repeated-dose regimen, monthly treatment of 1 mg/kg of DA-3091 is also expected to be suitable to suppress the body weight gain because the cumulative plasma concentration of exenatide is retained over effective plasma concentration.

In NAFLD mice, the effective plasma concentration and any effects of exenatide are not yet reported. Hence, we assessed the effect of biweekly treatment of two doses, 1 mg/kg and 2 mg/kg, of DA-3091 for 8 weeks. The representative markers of metabolic diseases like body weight gain, liver mass, hepatic injury, hepatic fat accumulation, glucose level and LDL-cholesterol level were well regulated under both doses regimens. Through these results, we proved that even 1 mg/kg of DA-3091 is sufficient to well control the representative markers of metabolic diseases. Both doses of DA-3091 significantly reduced the serum endotoxin level, consistent with the report that the endotoxin-induced activation of hepatic Kupffer cells resulting from intestinal bacterial overgrowth and increased intestinal permeability is a key factor in the onset of fructose-induced NAFLD (Cani et al. 2008; Brun et al. 2007).

Taken together of the anti-diabetic effects and newly observed effects in this study, it is demonstrated that DA-3091 has the potency as a drug for the treatment of metabolic disease.

## 4. Experimental

### *4.1. Animals*

Male C57BL/6 mice (6 weeks old) were purchased from Orient (Korea). Mice were housed individually in a room under controlled temperature (21–25 °C), humidity (40–70%), and lighting (12 hr light and dark cycle) with free access to water and a standard diet (13.5% fat calorie, Lab diet #5001) for 1 week. All animal experiments were conducted after the approval of the Institutional Animal Care and Use Committee (IACUC), which were also performed in accordance with the institutional "Standard Operation Procedure for Animal Care and Experiments" (SOP-ANC) of the Dong-A Pharm. Co. Ltd., and with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

### *4.2. DA-3091 preparation*

DA-3091 comprises sustained-release exenatide microspheres, which were prepared using oil-in-water (o/w) emulsion solvent evaporation. PLGA (poly(lactic-co-glycolic acid)) was used as the release modulator. Briefly, 10 mg of exenatide were dissolved in 1.0 mL of dichloromethane and 1.0 mL of methanol containing 300 mg of PLGA. The exenatide solution (oil) was emulsified in continuous phase (W: 1% PVA in water for injection) at a W:O volume ratio of 1:20 by homogenization at 3,000 rpm for 1 min (Lab mixer, Silverson, MA, USA). After emulsification, dichloromethane and methanol were evaporated by stirring at 37 °C for 3 h. The resultant microspheres were collected by centrifugation, washed three times with water for injection, and freeze dried (Kwak et al. 2009). In the animal studies, DA-3091 was suspended with vehicle [1.5% Sodium carboxy methyl cellulose (Na CMC), 0.87% sodium chloride (NaCl) (Sigma, MO, USA) and 0.1% Tween 20]. Sibutramin, a drug for obesity, was provided by Cipla Ltd.(India).

### *4.3. High fat diet (HFD)-induced obese mice and experimental protocol*

After a 1-week acclimation, mice were randomly divided into five groups of 10 mice per group. One group was fed standard diet and the other four groups were fed HFD (60% fat calorie, Research diet #D12492) for 4 weeks in order to induce obesity. Then, DA-3091 (1 or 2 mg/kg) was monthly subcutaneous administrated to two groups of HFD-fed groups for the following 8 weeks and sibutramin (10 mg/kg) was daily oral administered to one group of HFD-fed groups for the following 8 weeks. The detailed combination of diet and treatment in each five group was as follows: group 1; standard diet and vehicle, group 2; HFD and vehicle, group 3; HFD and DA-3091 (1 mg/kg/4-week), group 4; HFD and DA-3091 (2 mg/kg/4-week), group 5; HFD and sibutramin (10 mg/kg/day). Body weight and food intake were measured three times per week.

### *4.4. High sucrose diet (HSD)-induced non-alcoholic fatty liver disease (NAFLD) mice and experimental protocol*

After a 1-week acclimation, mice were randomly divided into four groups of 10 mice per group. One group was fed standard diet and the other three groups were fed HSD (10.5% fat calorie, 73% carbohydrate calorie and research diet #D12329) for 6 weeks in order to induce NAFLD. Then, DA-3091 (1 or 2 mg/kg) was biweekly subcutaneously injected to two groups of HSD-fed groups for the following 8 weeks. The detailed combination of diet and treatment in each four groups was as follows: group 1; standard diet and vehicle, group 2; HSD and vehicle, group 3; HSD and DA-3091 (1 mg/kg/2-week), group 4; HSD and DA-3091 (2 mg/kg/2-week).

### *4.5. Measurement of representative markers of hepatic injury and cholesterol level*

In HSD-induced NAFLD mice, serum alanine aminotransferase (ALT) and the aspartate aminotransferase (AST) levels were measured using an automated blood analyzer (KONELAB 20i, LONELAB). Also, the serum

levels of total cholesterol, high-density lipoprotein (HDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol were measured with the same automated blood analyzer.

### *4.6. Liver histological evaluation*

After treatment of vehicle and DA-3091 to HSD-induced NAFLD mice for 8 weeks, a cross section of the left lateral lobe of the liver of was fixed in 10% neutral buffered formalin. The liver tissues were dehydrated and embedded in paraffin, sectioned at 4 μm. The liver sections were stained with hematoxylin and eosin (HE) using an automated staining machine (Autostainner XL, Leica). The HE-stained sections were scanned using a microscope (Axioskop 2, Zeiss) and quantitative evaluation. Also, collagen fibers of the liver sections were stained with Masson Trichrome stain kit (Sigma, HT15).

### *4.7. Serum glucose level and endotoxin level*

After treatment with vehicle and DA-3091 to HSD-induced NAFLD mice for 8 weeks, non-fasting serum glucose levels were measured using AccuCheck Active (Roche Diagnostics, Germany). Also, at the end of experiment, serum samples were diluted and heated at 70 °C for 10 min to determine serum endotoxin levels using a Limulus Amebocyte Lysate (LAL) kinetic-GCL (Cambrex, 50-650U) (Cani et al. 2007).

### *4.8. Statistics*

The data are presented as a mean ± SEM. The statistical significance was determined by SigmaStat Software (SPSS inc. USA). Group comparisons were determined using Student's *t*-test or the one-way ANOVA test followed a Tukey *post-hoc* test. Differences between treatments were considered to be statistically significant at $p < 0.05$.

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