Pharmaceutical Research, Vol. 26, No. 11, November 2009 (© 2009)
DOI: 10.1007/s11095-009-9966-3

Research Paper

Pharmacokinetics and Efficacy of a Biweekly Dosage Formulation of Exenatide in Zucker Diabetic Fatty (ZDF) Rats

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Received June 26, 2009; accepted August 25, 2009; published online September 11, 2009

Purpose. To develop an improved sustained-release (SR) formulation of exenatide (a therapy for patients with type 2 diabetes mellitus) in a biweekly dosage form with therapeutic efficacy comparable to that achieved with twice-daily injections of the drug.
Methods. A SR formulation of exenatide, DA-3091, was prepared by single-emulsion solvent evaporation using poly(D,L-lactide-co-glycolide). Plasma exenatide, as well as plasma insulin, non-fasting blood glucose and HbA1c concentrations, and changes in food intake and body weight were evaluated in both Zucker diabetic fatty (ZDF) and ZDF lean control rats.
Results. After a single SC administration of DA-3091 (i.e., 2 mg/kg of exenatide), the plasma exenatide concentration increased and remained elevated in both groups. The concentrations of non-fasting blood glucose and HbA1c decreased significantly following a single SC injection of DA-3091 only in ZDF rats, indicating that the effects of exenatide are dependent on blood glucose concentration. On the other hand, both food intake and body weight gain were reduced in ZDF and ZDF lean control rats. A single injection of DA-3091 (i.e., 2 mg/kg of exenatide) lowered non-fasting blood glucose and HbA1c concentrations more effectively than 14 days of twice-daily administration of exenatide (i.e., 1.96 mg/kg of exenatide).
Conclusion. DA-3091 has the potential to be used safely and efficaciously in a biweekly dosing regimen.

KEY WORDS: exenatide; GLP-1 agonist; peptide delivery; poly(D,L-lactide-co-glycolide); sustained-release formulation.

INTRODUCTION

Incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, are gut-derived hormones that increase insulin secretion following their release into circulation. Currently, incretin mimetics, which mimic the actions of incretins, are receiving attention as a novel class of antidiabetic therapeutic agents. Among them, GLP-1 elicits glucoregulatory effects, including enhancement of glucose-dependent insulin secretion, suppression of excessive glucagon secretion, delayed gastric emptying, and appetite reduction (1, 2). However, exogenous GLP-1 administered via SC injection is rapidly inactivated by dipeptidyl peptidase-4 (DPP-4). As a result, the active form of GLP-1 has a very short half-life in circulation (<2 min) (3). Therefore, two strategies have been employed to overcome this limitation to the use of GLP-1 in the treatment of diabetes. The first is the use of GLP-1 receptor agonists that have prolonged half-lives due to their resistance to proteolytic degradation by DPP-4. The other strategy is inhibition of DPP-4 activity (4).

Exenatide (Byetta®, Eli Lilly), the first 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 (Fig. 1) (5). *In vitro*, exenatide is an agonist of pancreatic GLP-1 receptors (6). Reportedly, exenatide shares many glucoregulatory actions with GLP-1, including glucose-dependent insulinotropic, glucagonostatic, gastric slowing, and satiogenic effects (7). Moreover, exenatide promotes beta cell proliferation and islet neogenesis in rats (8). Exenatide has been approved by both the US (2005) and European Union (2006) as a therapy for patients with type 2 diabetes mellitus. Because exenatide has a longer half-life than GLP-1, it can be administered by twice-daily SC injection (9).

However, the complexity of the exenatide treatment regimen, including the frequency of administration and

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ABBREVIATIONS: AUC, the area under the plasma concentration time curve; C_{max}, the maximum plasma concentration; DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; HbA1c, glycosylated hemoglobin; PLGA, poly(D,L-lactide-co-glycolide); T_{max}, the time to reach the maximum concentration; ZDF, Zucker diabetic fatty.

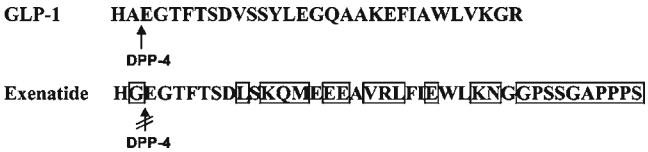

Fig. 1. Amino acid sequences of native GLP-1 and exenatide. Boxed letters indicate changes introduced in exenatide. The N-terminal dipeptide "HA" of GLP-1 is the site of proteolytic cleavage by DPP4. The marked arrow indicates the absence of DPP4 activity.

duration of treatment, negatively affects patient compliance. Reduction of the required frequency of administration is one strategy that might significantly enhance compliance. Eli Lilly has developed a sustained-release (SR) microsphere formulation of exenatide (Byetta® Long Acting Release, LAR), which is prepared using a poly(D,L-lactide-co-glycolide) (PLGA, e.g., RG503H) (10, 11), for once-weekly SC administration that is now being evaluated in a phase III clinical trial (12). However, since Byetta® LAR is prepared through a rather complicated process, i.e., a water-in-oil-in-oil (w/o/o) coacervation method, concerns exist regarding the production of exenatide-derived impurities during the preparation. In addition, the mean particle size of Byetta® LAR is about 50 μm, which necessitates the use of painfully large, 23-gauge needles for the SC administration (13). Moreover, the pharmacokinetic profile of Byetta® LAR is inadequate in SD rats (10), and the weekly dose of exenatide in Byetta® LAR (i.e., single dose of 2 mg/human) is much higher (14~28 fold) than that for Byetta® (i.e., 5~10 μg/human twice-daily, which corresponds to a total weekly dose of 70~140 μg/human). Improper release of exenatide from Byetta® LAR *in vivo* may explain the poor pharmacokinetics and the necessity of a much higher dose.

Therefore, the purpose of the present study was to develop an improved SR formulation of exenatide for biweekly SC administration with therapeutic efficacy comparable to that achieved with serial, twice-daily injections of exenatide for 14 days. The specific goal was to improve the potential drawbacks of Byetta® LAR. In the present study, exenatide was formulated into microspheres using PLGA RG502H (M.W. 8~12 KDa), a polymer with a lower molecular weight than that used for Byetta® LAR (i.e., PLGA RG503H, M.W. 30~64 KDa), with the hope that *in vivo* release of exenatide from the PLGA RG502H formulation would be more complete compared with release from the PLGA RG503H formulation. In addition, reduction of microsphere size was attempted in the present study so that the SC administration could be conducted using syringe needles that were smaller than 23-gauge. Lastly, development of a simplified manufacturing process was attempted to minimize the formation of exenatide-derived impurities during manufacture. A simple oil-in-water (o/w) emulsion solvent evaporation method was therefore adopted. Then, the feasibility of the clinical use of the developed formulation was evaluated. Plasma exenatide concentrations (pharmacokinetic markers), as well as plasma insulin, non-fasting blood glucose and glycosylated hemoglobin (HbA1c) concentrations, and changes in food intake and body weight (efficacy markers) were evaluated in both Zucker diabetic fatty (ZDF) and ZDF lean control rats following treatment with this novel formulation. The efficacy of the SR formulation was compared with that of twice-daily subcutaneous injections of an exenatide solution.

MATERIALS AND METHODS

Materials

Exenatide (American Peptide, CA, USA), poly(D,L-lactide-co-glycolide) (PLGA, RG502H, inherent viscosity 0.16~0.24 dl/g, M.W. 8~12 KDa, Boehringer-Ingelheim, Germany), poly vinyl alcohol (PVA, MW 30~70 KDa) (Merck, Germany), dichloromethane and methanol (Sigma, MO, USA) were used in the preparation of the SR-microspheres. Sodium carboxymethyl cellulose (Na CMC), Tween 20 and sodium chloride (NaCl) (Sigma, MO, USA), and phosphate-buffered saline (PBS, Cambrex, MD, USA) were used in the animal studies.

Animals

Male Zucker diabetic fatty (ZDF) rats [ZDF/Gmi-(fa/fa), 11~12 weeks old, weighing 395±6 g, Charles River, NY, USA] and male ZDF lean control rats [ZDF/Gmi-(+/fa), 11~12 weeks old, weighing 308±5 g, Charles River, NY, USA] were used in the present study. The animals were housed under standard conditions and a 12-hr light/dark cycle with free access to standard chow (Purina 5001, Purina Mills Inc., St. Louis, MO, USA) and water throughout the study period. The animals were acclimated for 1 week prior to beginning the experiment. Eighteen male ZDF rats were assigned to three treatment groups (n=6 per group) and fifteen male ZDF lean control rats were assigned to the same three treatments (n=5 per group). Mean HbA1c concentrations and body weights in the treatment groups were similar, ranging from 6.0% to 6.2% and 391–398 g, respectively, for the ZDF rats, and 3.5% and 304–316 g, respectively, for the ZDF lean control rats.

In the present study, all procedures involving experimental animals were consistent with the guidelines set by the National Institutes of Health (NIH publication no. 85-23, revised 1985) and were approved by the animal-use committee of the Dong-A Research Center.

Preparation of SR-Exenatide Microspheres, DA-3091

SR-exenatide microspheres were prepared using oil-in-water (o/w) emulsion solvent evaporation, as previously described (14), with appropriate modifications (Fig. 2). PLGA (RG502H) 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:oil volume ratio of 1:20 by homogenization at 3,000 rpm for 1 min (Lab mixer, Silverson, MA, USA). After emulsification, the 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. The microsphere formulation prepared in the present study was termed DA-3091.

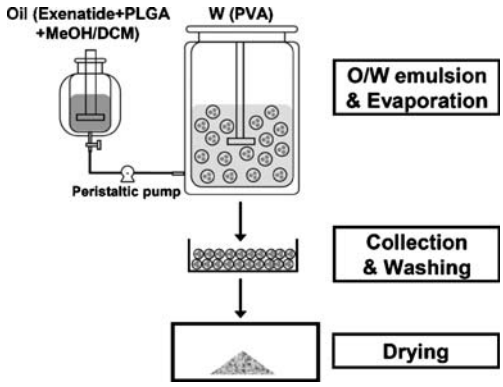

Fig. 2. Schematic illustration of the solvent evaporation method used to prepare the DA-3091 microspheres.

Physicochemical Characterization of DA-3091

The volume-weighted mean particle size of the microspheres was determined for an aqueous dispersion of DA-3091 (20 mg/500 mL) using a particle size analyzer (Master sizer 2000, Malvern, Worcestershire, UK). Morphological analysis was performed using scanning electron microscopy (SEM, Hitachi S-3500N, Tokyo, Japan) under a magnification of 300- or 2000-fold after coating the DA-3091 with Au-Pd (Hitachi E-1010, 25 KV, 120 s). For determination of exenatide content and purity in the formulation, DA-3091 microspheres were dissolved in acetonitrile (20 mg/mL). The content and purity were evaluated by high performance liquid chromatography (HPLC) using an HPLC system [C18 column ; Waters 2695 Separations Module; and, Waters 2996 PDA detector, Milford, MA, USA]. Aliquots of the acetonitrile solution were eluted with a mobile phase [0.1% trifluoroacetic acid (TFA) in distilled water : 0.1% TFA in acetonitrile = 60 : 40], and the eluate was monitored by UV detection at 210 nm.

Tests for sterility, bacterial endotoxin and residual solvent were also performed according to the respective Methods of Analysis in the European Pharmacopoeia (EP, 6th edition, 2006).

In Vitro Release Study

DA-3091 microspheres (30 mg) were suspended in 1.5 mL PBS (pH 7.4) containing 0.02% (*w*/*v*) Tween 20. The suspension was incubated at 37°C under continuous agitation (i.e., 30 rpm) for 21 days. At pre-determined intervals (e.g., 24 h), the microspheres and supernatant were separated by centrifugation at 3,000 g for 30 min. An aliquot (1.3 mL) of the supernatant was withdrawn and an equal volume of fresh release medium was added. The amount of exenatide in the collected supernatant was measured using a Micro BCA™ protein assay kit (Pierce, IL, USA).

Plasma Concentrations of Exenatide and Insulin in ZDF and ZDF Lean Control Rats

Exenatide pharmacokinetics and plasma insulin concentrations were assessed in male ZDF (n=6) and ZDF lean control (n=5) rats. The rats received a single subcutaneous injection of an aqueous suspension of DA-3091 (0.2%, *w*/*v*) via a 27-gauge needle at an exenatide dose of 2 mg/mL/kg. The homogeneous dispersion was prepared within 2 min by suspending the microspheres in an aqueous vehicle (0.87% NaCl, 1.5% Na CMC, 0.1% Tween 20). Blood samples (approximately 0.25 mL) were collected from the tail vein into Microtainer® tubes (BD, NJ, USA) containing K_2EDTA at the following time points: before exenatide injection; 1, 2, 4 and 8 h post-injection; and, 2, 4, 7, 9, 11, 14, 18, 21, 25 and 28 days post-injection. After centrifugation (i.e., 10,000 g, 5 min), plasma was collected and stored at −20°C until it could be analyzed for exenatide and insulin.

Plasma concentrations of exenatide were measured using the Exendin-4 EIA kit (EK-070-94, Phoenix pharmaceuticals, CA, USA). The exenatide standard provided in the ELISA kit was serially diluted using 10% rat plasma to minimize plasma interference. Likewise, the samples from ZDF and lean control rats were diluted 1/10 with assay buffer and subsequently analyzed. Plasma concentrations of insulin were measured using a rat insulin ELISA kit (Mercodia, Sweden). The lower limit of detection for both the exenatide and insulin ELISA was 0.07 ng/mL.

Efficacy Study in ZDF and ZDF Lean Control Rats

The efficacy of DA-3091 was assessed in male ZDF and ZDF lean control rats. Eighteen male ZDF rats and fifteen male ZDF lean control rats were assigned to three treatment groups (n=6 per group for ZDF rats and n=5 per group for ZDF lean control rats): exenatide solution (twice daily SC injection), DA-3091 (single SC injection), or the aqueous vehicle (single SC injection).

Exenatide solution-treated groups received twice-daily subcutaneous injections of exenatide in PBS at 10:00 A.M. and 5:00 P.M. via a 27-gauge needle at an exenatide dose of 70 μg/mL/kg for 14 days. Only the second injection on the first day was administered at 6:00 P.M. to allow for measurement of non-fasting blood glucose 8 h after the first injection. The first administration was performed on Monday.

The DA-3091-treated groups received a single subcutaneous injection of an aqueous suspension of DA-3091 via a 27-gauge needle at an exenatide dose of 2 mg/mL/kg at 10:00 A.M. on Monday. The aqueous suspension of DA-3091 was prepared as described above. The exenatide dose of the DA-3091 was 2 mg/kg, which was nearly equivalent to the total exenatide dose provided by twice-daily administration of exenatide solution at an exenatide dose of 70 μg/kg for 2 weeks (i.e., total exenatide dose of 1.96 mg/kg).

The aqueous vehicle-treated groups received a single subcutaneous injection of the aqueous vehicle via a 27-gauge needle at a volume of 1 mL/kg at 10:00 A.M. on Monday.

On the first day of the study, non-fasting blood glucose concentrations were measured before exenatide injection, and at 1, 2, 4, 6, 8 and 24 h post-injection, using AccuCheck Active (Roche Diagnostics, Germany). Subsequently, non-fasting blood glucose was measured three times weekly (i.e., Monday, Wednesday and Friday at 10:00 A.M.). HbA1c concentrations in blood samples collected from the tail vein (10 μL) were measured weekly (Mondays) using a DCA 2000 analyzer (Bayer Diagnostics, NY, USA). Food intake of all animals housed together in one cage (two cages for ZDF rats and one cage for ZDF lean control rats) was measured three

times weekly (i.e., Monday, Wednesday and Friday at 10:00 A.M.), and average food intake per each rat was calculated by dividing the intake of all animals in one cage by the number of animals per cage. Body weight was also measured three times per week at 10:00 A.M.

Pharmacokinetic Analyses and Statistics

The maximum plasma concentration (C_{max}) of exenatide and the time to reach the maximum concentration (T_{max}) were determined directly from the data. Non-compartmental pharmacokinetic analyses were performed on plasma exenatide concentrations *vs*. time profiles using WinNonLin (Pharsight Corporation, Mountain View, CA, USA). The area under the plasma concentration time curve (AUC) from time zero to the last day of sampling (AUC_{0-28d}) was calculated using a standard method (15).

All results from the *in vivo* experiments are reported as means ± S.E. Statistical analyses were carried out using SigmaStat Software (SPSS Inc. Chicago, IL, USA). Group comparisons were performed using *t*-tests or one-way ANOVA with a Tukey *post-hoc* test. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

Physicochemical Characteristics of DA-3091 Microspheres

The mean particle size of the DA-3091 microspheres was approximately 20 μm, which was much smaller than that of Byetta® LAR (i.e., approximately 50 μm). As a result of the injectability test, DA-3091 could be administered via a 27-gauge needle. The SEM images showed that SR-exenatide microspheres were hollow spheres with a smooth surface (Fig. 3). The exenatide content in DA-3091 was approximately 3% (*w*/*w*), and the encapsulation efficiency was 87±3%. The chromatographic profiles of DA-3091 for exenatide were identical to those of the exenatide used in preparation of the microspheres (data not shown), suggesting that exenatide-derived impurities, such as those resulting from either oxidation or deamidation, were not formed during manufacture of the DA-3091. Apparently, the lack of impurities was due to the simplicity of the manufacturing process. Thus, DA-3091 microspheres were prepared without significant reductions in exenatide content or purity.

The results of both sterility and endotoxin tests confirmed the production of a sterile DA-3091 formulation. The residual solvent (i.e., dichloromethane and methanol) content of the DA-3091 was less than the solvent concentration limits in EP (6th edition, 2.4.24).

In Vitro Release of Exenatide from DA-3091 Microspheres

Figure 4 shows the cumulative release of exenatide from DA-3091 microspheres *in vitro*. The release was very slow until day 5 of the experiment, at which time the polymer apparently started to dissolve and become degraded. After 5 days, the rate of release was relatively constant. After 21 days, exenatide release was complete, implying possible good release of exenatide *in vivo*. These results indicate that the release of exenatide follows near zero-order kinetics without an initial burst.

Plasma Concentrations of Exenatide and Insulin in DA-3091-Treated ZDF and ZDF Lean Control Rats

In ZDF rats treated with DA-3091 at an exenatide dose of 2 mg/kg, plasma exenatide concentrations increased rapidly, reaching the first peak within one hour, followed by a rapid decline on day 1 (inset of Fig. 5A), and then by a gradual increase and substantial sustainment for 28 days, as shown in Fig. 5A. T_{max} was determined to be 9.3 days, and C_{max} was 8.2 ng/ml for ZDF rats (Table I). The initial rapid increase seemed to result from release of the drug from the surface of DA-3091, while the second gradual increase was attributed to sustained release of exenatide from the inside of microspheres. In ZDF lean control rats, a similar profile was observed with a T_{max} of 8.2 days and a C_{max} of 7.9 ng/ml. There were no significant differences in the plasma concentration of exenatide between the ZDF and ZDF lean control rats. These results indicate that the release of exenatide from DA-3091 was fairly well-sustained in both ZDF and ZDF lean control rats. Therefore, the poor pharmacokinetics of exenatide from Byetta® LAR, which exhibited low serum exenatide levels (i.e., trough) between days 4 and 17 (10), were improved.

In ZDF rats, the plasma concentration of insulin increased immediately following DA-3091 treatment and remained elevated for 28 days, as shown in Fig. 5B. However, insulin concentrations remained unchanged following DA-3091 injection in ZDF lean control rats. It is interesting to

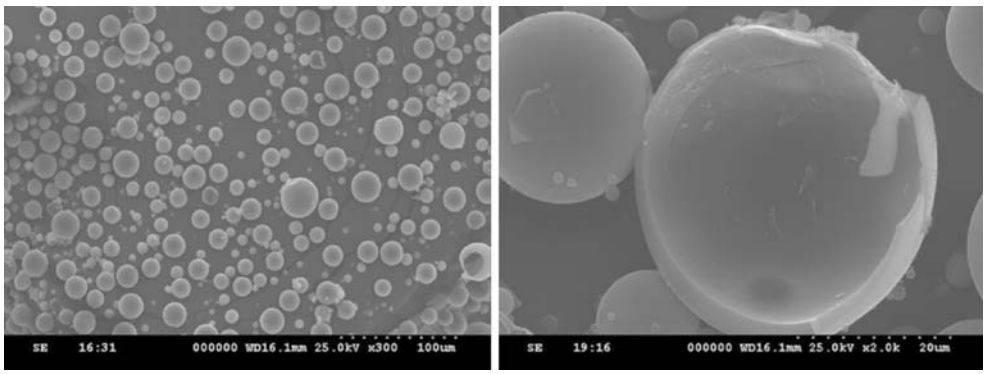

Fig. 3. Scanning electron micrographs of the surface morphology (*left*, X 300) and cross-section (*right*, X 2000) of the DA-3091 microspheres.

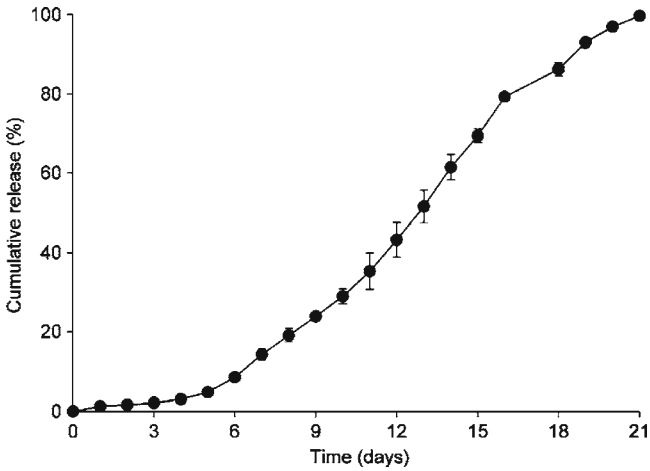

Fig. 4. *In vitro* exenatide release from DA-3091 microspheres in PBS (pH 7.4) containing 0.02% (*w*/*v*) Tween 20 at 37°C. Data are means ± SE (n=3).

note that the concentration of insulin increased continuously after 9.3 days (Fig. 5B), despite the rapid decrease in exenatide concentrations during this period (Fig. 5A). This apparent inconsistency may be explained by the fact that exenatide does not accelerate insulin secretion in a blood-exenatide-dependent manner, but in a blood-glucose-dependent manner, as reported previously by Parkes *et al.* (16). In ZDF lean control rats, insulin concentrations remained unchanged following DA-3091 injection, confirming that exenatide accelerates insulin secretion in a blood-glucose-dependent manner.

Efficacy of DA-3091 in ZDF and ZDF Lean Control Rats

Non-fasting Blood Glucose and HbA1c Concentrations

In ZDF rats, twice daily administration of exenatide solution at an exenatide dose of 70 μg/kg for 14 days had no effect on non-fasting blood glucose concentrations, except at 1, 2, 4, 8 h after the first injection and after 7 days of treatment compared with the aqueous vehicle administration, as shown in Fig. 6A. However, a single injection of DA-3091 at an exenatide dose of 2 mg/kg significantly decreased non-fasting glucose compared with injection of aqueous vehicle for 3 weeks. These results showed that, at a nearly equivalent exenatide dose, a single injection of DA-3091 (i.e., 2 mg/kg of exenatide) lowered non-fasting blood glucose concentrations more effectively than 14 days of twice-daily administration of an exenatide solution (i.e., 1.96 mg/kg of exenatide). By contrast, in ZDF lean control rats with normal blood glucose concentrations, neither exenatide in solution or in DA-3091 microspheres affected non-fasting blood glucose compared with the aqueous vehicle (Fig. 6B). This result demonstrates that the effects of exenatide are dependent on blood glucose concentration.

Table I. Pharmacokinetic Parameters of Exenatide in ZDF and ZDF Lean Control Rats Following a Single SC Injection of DA-3091 at an Exenatide Dose of 2 mg/kg

	ZDF rats (n=6)	ZDF lean control rats (n=5)
T_{max} (day)	9.3±0.33	8.2±0.49
C_{max} (ng/mL)	8.2±1.3	7.9±2.4
AUC_{0-28d} (ng·day/mL)	55.3±6.25	51.5±6.66

It is well-known that HbA1c is an indirect indicator of cumulative blood glucose concentrations because it results from non-enzymatic irreversible glycation. Thus, HbA1c is a more sensitive index of glycemic control than glucose concentration, which is highly variable (17). As shown in Fig. 7A, administration of exenatide solution had no effect on HbA1c compared with the aqueous vehicle in ZDF rats. However, DA-3091 treatment significantly decreased HbA1c concentrations for 7 weeks following administration in ZDF rats. Because glycation of hemoglobin occurs continuously throughout the life-span of an erythrocyte, which is approximately 53–60 days in the rat (18), both the time required to reach a steady-state HbA1c concentration and the duration of the effects of DA-3091 on HbA1c concentration were longer than those observed for non-fasting blood glucose concen-

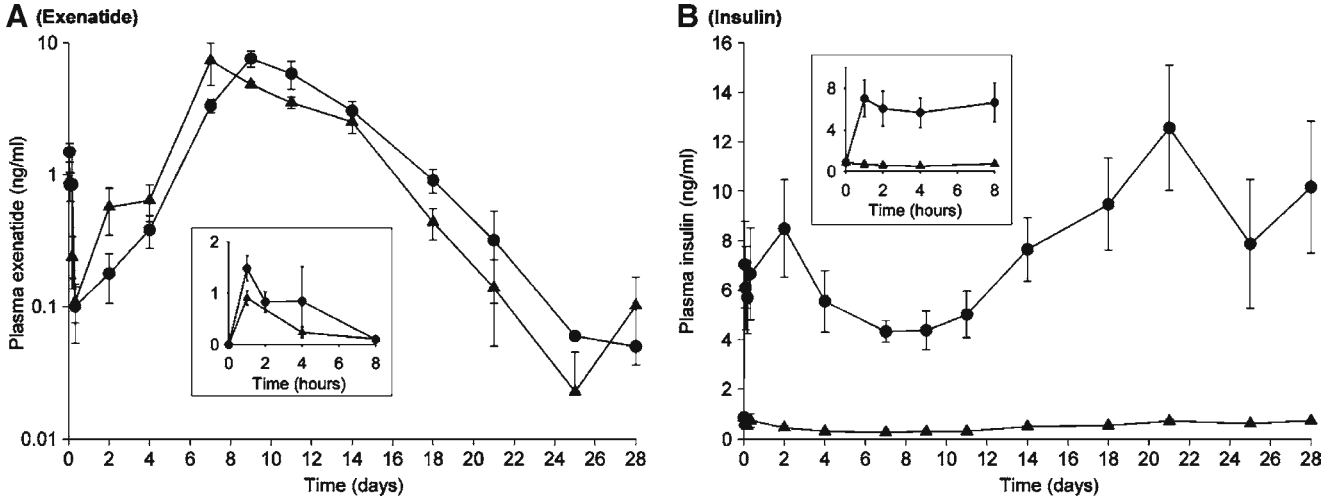

Fig. 5. Average plasma exenatide (A) and insulin (B) concentrations in ZDF (●, n=6) and ZDF lean control (▲, n=5) rats treated with DA-3091 at an exenatide dose of 2 mg /mL/kg. Data are means ± SE.

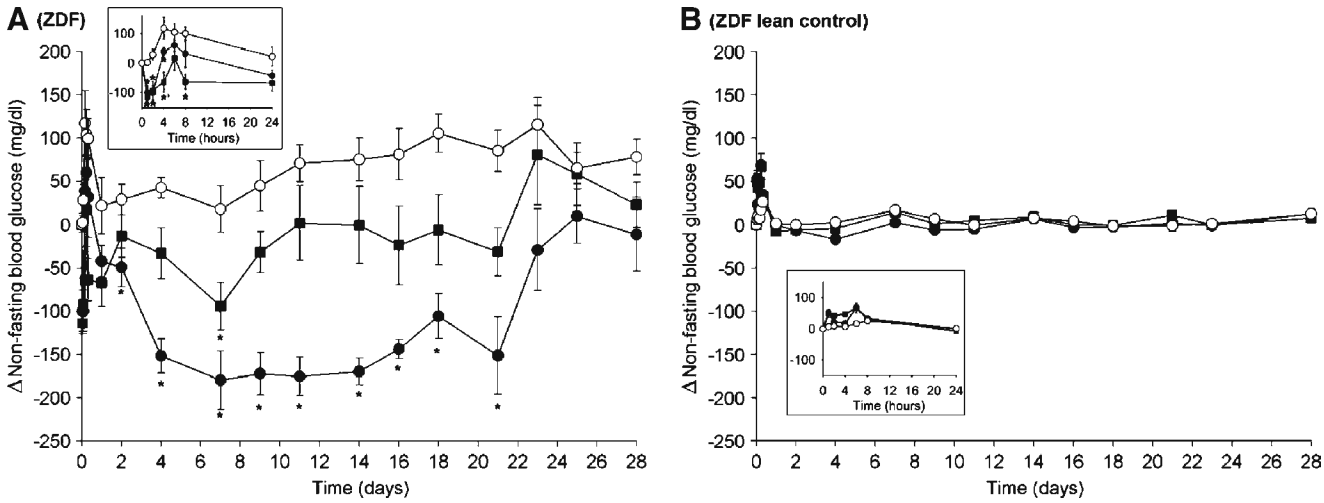

Fig. 6. Change in non-fasting blood glucose concentrations in ZDF (A, n=6) and ZDF lean control (B, n=5) rats treated with exenatide solution (■), DA-3091 (●) or the aqueous vehicle (○). Exenatide solution was administered SC twice daily at an exenatide dose of 70 μg/mL/kg for 14 days. DA-3091 was administered as a single SC injection at an exenatide dose of 2 mg/mL/kg. The aqueous vehicle was administered as a single SC injection at a volume dose of 1 mL/kg. Data are means ± SE. *: $p<0.05$, *vs.* the aqueous vehicle.

tration (Fig. 6A). In addition to the effects on non-fasting blood glucose, the HbA1c results also confirmed that a single injection of DA-3091 (at an exenatide dose of 2 mg/kg) is more effective than 14 days of twice-daily injection of exenatide solution (at an exenatide dose of 70 μg/kg).

The lesser effect of exenatide solution on both non-fasting blood glucose and HbA1c appears to be associated with timing of blood collection. Blood was sampled just prior to administration of exenatide solution (i.e., 10:00 A.M.). In our preliminary study, the concentration of exenatide in the plasma of ZDF rats following a single SC injection of exenatide solution at an exenatide dose of 70 μg/mL/kg, rapidly reached a C_{max} of 45.3 ng/ml at a T_{max} of 1.3 h, and then decreased rapidly with a half-life of 3~5 h. Therefore, at the time of blood sampling following twice daily administration of exenatide solution, the plasma concentration of exenatide was likely very low, resulting in only marginal efficacy. On the other hand, in ZDF lean control rats with normal blood glucose concentrations, there was no significant change in HbA1c concentration after administration of either exenatide solution or DA-3091 compared with the aqueous vehicle (Fig. 7B). This result also indicates that the effects of exenatide, especially of the DA-3091 formulation, are dependent on blood glucose concentration.

Food Intake and Body Weight

As shown in Fig. 8A, administration of either exenatide solution or DA-3091 reduced food intake in ZDF rats, although the reduction in food intake in the DA-3091 group was greater than that in the exenatide solution group. Unlike the results observed for non-fasting blood glucose and HbA1c concentrations, food intake was reduced in ZDF lean control rats after administration of either exenatide solution or DA-

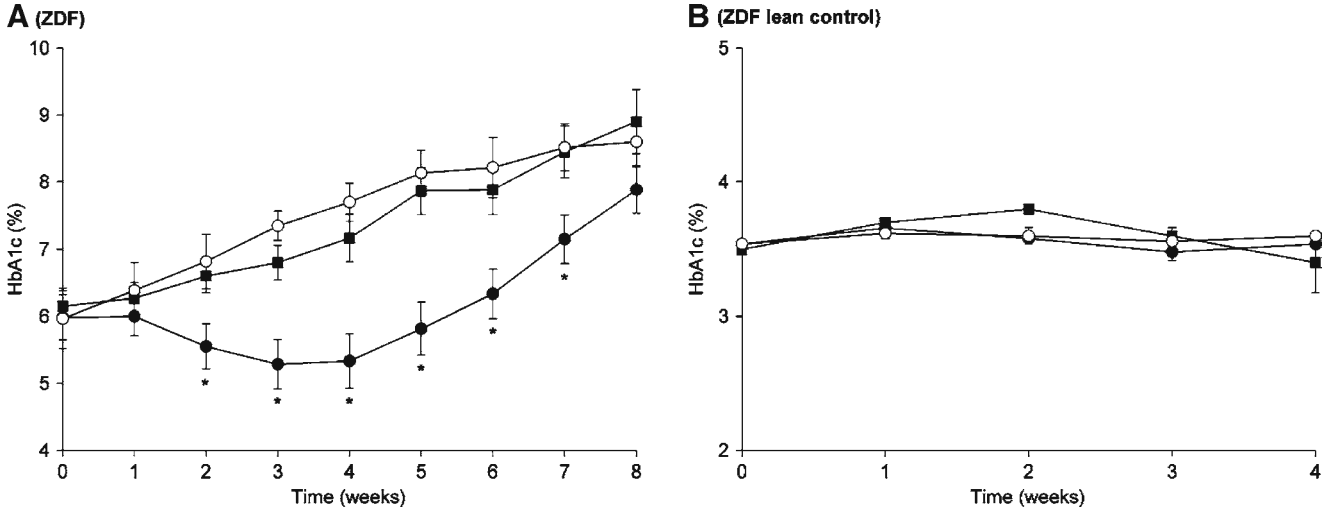

Fig. 7. Change in HbA1c concentrations in ZDF (A, n=6) and ZDF lean control (B, n=5) rats treated with exenatide solution (■), DA-3091 (●) or the aqueous vehicle (○). Exenatide solution was administered SC twice daily at an exenatide dose of 70 μg/mL/kg for 14 days. DA-3091 was administered as a single SC injection at an exenatide dose of 2 mg/mL/kg. The aqueous vehicle was administered as a single SC injection at a volume dose of 1 mL/kg. Data are means ± SE. *: $p<0.05$, *vs.* the aqueous vehicle.

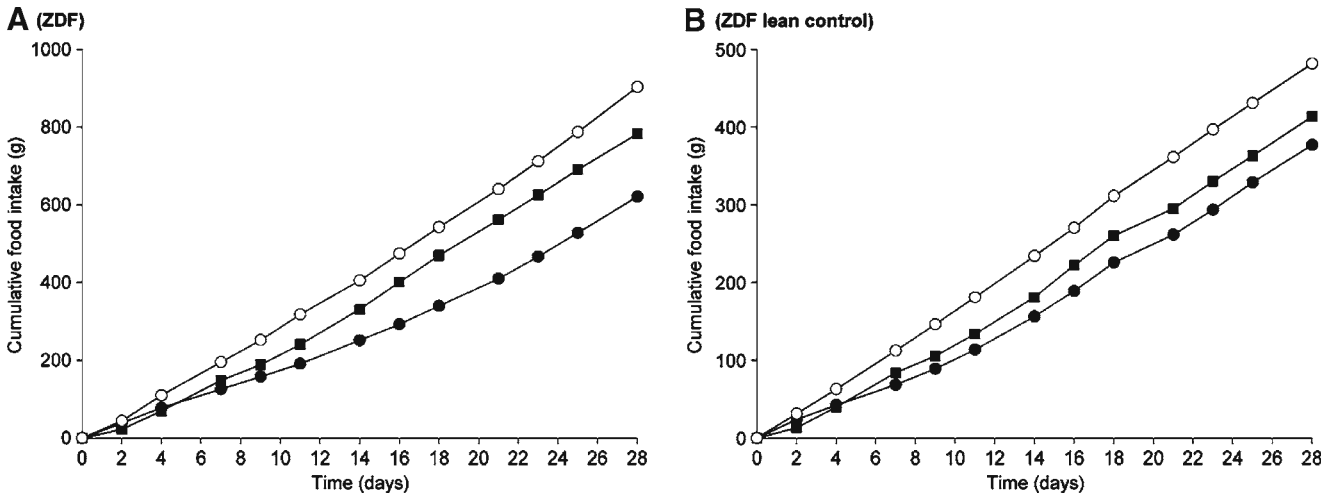

Fig. 8. Cumulative food intake in ZDF (A) and ZDF lean control (B) rats treated with exenatide solution (■), DA-3091 (●), or the aqueous vehicle (○). Exenatide solution was administered SC twice daily at an exenatide dose of 70 μg/mL/kg for 14 days. DA-3091 was administered as a single SC injection at an exenatide dose of 2 mg/mL/kg. The aqueous vehicle was administered as a single SC injection at a volume dose of 1 mL/kg. Food intake was recorded as the mean of six (A) and five (B) rats.

3091 (Fig. 8B). This result suggests that exenatide has anorectic effects in both ZDF and ZDF lean control rats, consistent with the results of a previous study (19).

As shown in Fig. 9A, body weight gain was suppressed after administration of either exenatide solution or DA-3091 in ZDF rats. In the aqueous vehicle group, body weight increased as a function of time (i.e., in proportion with disease progression). As a result, body weight in rats treated with either exenatide solution or DA-3091 was significantly lower than that of the aqueous vehicle-treated rats. Unlike other markers of efficacy examined in the present study, such as non-fasting blood glucose and HbA1c, there was no significant difference in body weight gain between the exenatide- and DA-3091-treated groups. Similar to the results observed for food intake, body weight gain was reduced after administration of either exenatide solution or DA-3091 even in ZDF lean control rats. The effect of DA-3091 on body weight gain was greater than that of exenatide solution on days 4 to 18 of the study (Fig. 9B).

DISCUSSION

A sustained-release microsphere formulation of exenatide was prepared by a simple o/w emulsion solvent evaporation method using PLGA as the release modifier. Due to the simplicity of the preparation method, generation of exenatide-derived impurities during manufacturing was minimal. Indeed, no impurities were detected in the DA-3091 preparations. In general, drugs formulated in polymeric devices are released by either diffusion through the polymer barrier, degradation of the polymer, or a combination of both diffusion and degradation mechanisms (20). A similar mechanism might be responsible for the release of exenatide from DA-3091. It is well-known that PLGA polymers with a 50:50

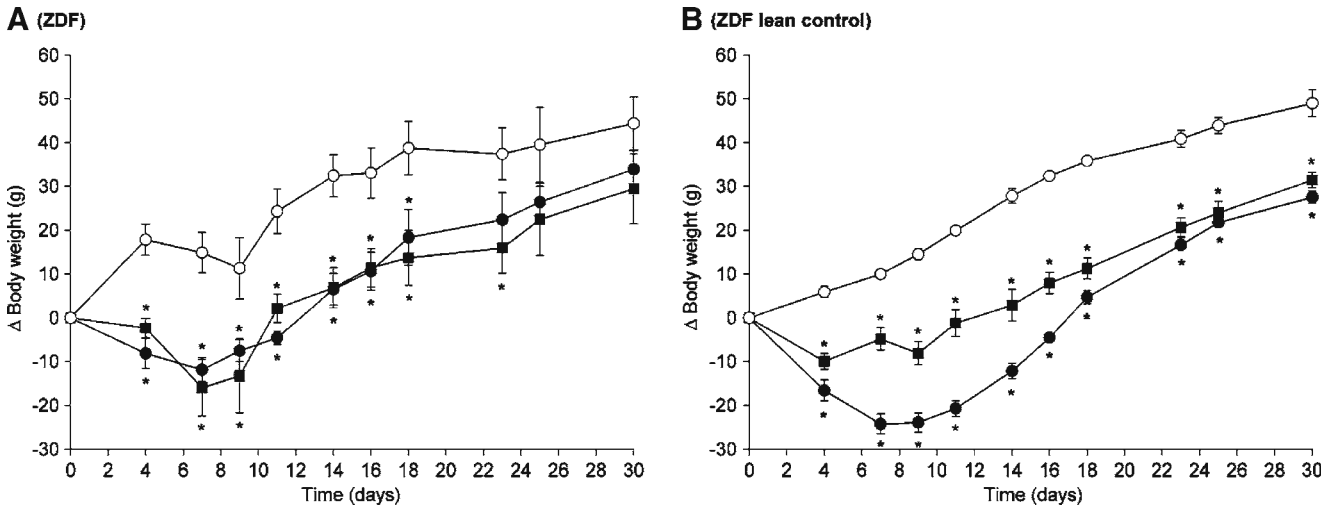

Fig. 9. Change in body weight in ZDF (A, $n=6$) and ZDF lean control (B, $n=5$) rats treated with exenatide solution (■), DA-3091 (●), or the aqueous vehicle (○). Exenatide solution was administered SC twice daily at an exenatide dose of 70 μg/mL/kg for 14 days. DA-3091 was administered as a single SC injection at an exenatide dose of 2 mg/mL/kg. The aqueous vehicle was administered as a single SC injection at a volume dose of 1 mL/kg. Data are means ± SE. *: $p<0.05$, *vs.* the aqueous vehicle.

ratio of lactide to glycolide degrade most rapidly, and low-molecular weight polymers degrade more rapidly than higher molecular weight polymers (21). Therefore, faster and more complete release of exenatide is expected from DA-3091, a formulation prepared using the low-molecular weight polymer PLGA (50:50, RG502H), than from Byetta®-LAR, for which the high-molecular weight polymer PLGA RG503H was used. As expected, *in vitro* release of exenatide from DA-3091 microspheres was complete within 21 days, following near zero-order release kinetics without an initial burst (Fig. 4). Although the initial rate of exenatide release *in vitro* was somewhat slow, *in vivo* plasma exenatide and insulin concentrations increased immediately after administration of DA-3091. A similar phenomenon has been reported for tetanus toxoid-containing PLGA microspheres, indicating that release of drugs from microspheres occurs more rapidly *in vivo* than *in vitro* (22). This report supports fairly good release of exenatide from DA-3091 *in vivo*. In addition, SC administration of DA-3091 is feasible using a 27-gauge syringe needle owing to the smaller microspheres (i.e., 20 μm) compared with Byetta®-LAR (i.e., 50 μm), which might improve patient tolerance of the injection and thereby increase patient compliance.

To assess the potential clinical use of the sustained-release DA-3091 formulation, preclinical studies were conducted in ZDF and ZDF lean control rats. As shown in Fig. 5, the plasma concentration of exenatide increased and remained elevated following DA-3091 administration in both ZDF and ZDF lean control rats. Therefore, DA-3091 appears superior again in terms of pharmacokinetics compared to Byetta® LAR, which demonstrated an inadequate pharmacokinetic profile with a trough. Moreover, the insulinotropic effect, i.e., the ability to amplify glucose-stimulated insulin secretion without increasing insulin secretion at low plasma glucose concentrations, was confirmed. The glucose-dependence of exenatide action offers the advantage of increased drug safety compared with agents that increase insulin secretion via glucose-independent mechanisms (e.g., sulfonylureas), suggesting that DA-3091 can be used clinically without the risk of hypoglycemia.

Figures 6 and 7 show that non-fasting blood glucose and HbA1c concentrations were significantly decreased after a single injection of DA-3091 only in ZDF rats, also demonstrating the blood glucose-dependent effects of exenatide. Non-fasting blood glucose concentrations were significantly reduced for 3 weeks following the injection (Fig. 6A). The prolonged effect may occur because plasma exenatide is maintained at concentrations greater than EC_{50} (52 pmol) for 3 weeks (Fig. 5A) following DA-3091 treatment, as has also been reported for Byetta®-LAR (11). The effects of DA-3091 on HbA1c were observed for longer post-treatment because HbA1c is an indicator of cumulative blood glucose concentration.

Unlike glucose and HbA1c levels, food intake and body weight gain were reduced after administration of either exenatide solution or DA-3091 in both ZDF and ZDF lean control rats (Figs. 8, 9). Exenatide has anorectic effects due to its action on the hypothalamus to increase satiety and delay gastric emptying (23).

Taken together, the results of the present study suggest that, for a nearly equivalent exenatide dose, the efficacy of a single injection of DA-3091 (exenatide dose: 2 mg/kg) might be superior to that obtained with twice daily periodic injections of exenatide solution for 2 weeks (exenatide dose: 70 μg/kg per each injection, total dose: 1.96 mg/kg) in the ZDF rat model of type 2 diabetes. It should be noted that an exenatide dose 14~28-fold greater than that used for twice-daily administration was necessary for Byetta® LAR to elicit comparable efficacy (i.e., 2 mg/human *vs*. 70~140 μg/human). Improper *in vivo* release of exenatide from Byetta® LAR might be responsible for the greater required dose. The higher molecular weight of PLGA (RG503H) used for Byetta® LAR might be the mechanism for incomplete *in vivo* release of the drug.

In conclusion, a simple method for the preparation of a biweekly SR exenatide formulation was developed. Microspheres small enough to be administered subcutaneously through a 27-gauge needle (i.e., 20 μm) without contamination by exenatide-derived impurities were prepared. Treatment with DA-3091 prolonged the increase in plasma exenatide and insulin concentrations, continuously decreased non-fasting blood glucose and HbA1c concentrations, and suppressed food intake and body weight gain. The results of these preclinical studies demonstrate the excellent pharmacokinetic profile and efficacy of DA-3091. In summary, DA-3091 has the potential to be clinically effective when administered once biweekly, and thus promises significantly improved patient compliance.

ACKNOWLEDGMENTS

This work was supported by the Ministry for Health, Welfare and Family Affairs, (A080017) and by the Ministry of Science and Technology through the National Research Laboratory Program (ROA-2006-000-10290-0), Republic of Korea.

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