

## RESEARCH PAPER

# Site-specific PEGylation of exenatide analogues markedly improved their glucoregulatory activity

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## BACKGROUND AND PURPOSE

Exenatide is a 39-amino-acid peptide widely used to manage type 2 diabetes mellitus. However, it has a short plasma half-life and requires a twice daily injection regime. To overcome these drawbacks we used maleimide-polyethylene glycol to induce site-specific PEGylation.

## EXPERIMENTAL APPROACH

The analogue PB-105 (ExC39) was produced by replacing cysteine at position 39 of exenatide to provide a free thiol group. PB-105 showed the same glucoregulatory activity as exenatide in mice. Site-specific PEGylation of PB-105 was performed to produce PB-110 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC39PEG40kDa). Their effects on intracellular cAMP, acute glucoregulatory activity and pharmacokinetic profile were compared in mice and rats.

## KEY RESULTS

PEGylation shifted the concentration–response curve of PB-105 to the right in a parallel, polyethylene glycol mass-dependent manner but with an inflexion point of at least 20 kDa. The activities of PB-107 and PB-108 but not PB-106 were reduced by 90% and 99%. PEGylation affected *in vivo* glucoregulatory activity in the same 'Inflexion-Shift' fashion at least at 20 kDa, but linearly increased plasma duration and systemic exposure without inflexion. PB-106 had a plasma  $t_{1/2}$  approximately 10-fold that of PB-105, and exhibited superior glucoregulatory activity compared with PB-105 in normal and diabetic mice.

## CONCLUSIONS AND IMPLICATIONS

Site-specific PEGylation of exenatide with a permanent amide linkage affects its activity in a new type of 'Inflexion-Shift' fashion. PB-106 is a putative new analogue for treating diabetes; it possesses no loss of *in vitro* activity, prolonged plasma duration and superior, improved *in vivo* glucoregulatory activity compared with exenatide.

## Abbreviations

AAC, area above lowering blood glucose time course curve; AUC, area under time course curve; GLP-1, glucagon-like peptide-1; PEG, polyethylene glycol; STZ, streptozotocin;  $t_{1/2}$ , half recovery time

## Introduction

It is known that gut-derived mammalian incretins, such as glucagon-like peptide-1 (GLP-1), regulate blood glucose levels following their release into the circulation. GLP-1 exhibits glucoregulation via activation of GLP-1 receptors, located in pancreatic periductal cells,  $\alpha$ - and  $\beta$ -cells, as well as the kidney, heart, stomach and brain (Holst, 2007), to increase

glucose-dependent insulin secretion, which decreases risks of hypoglycaemia (Parkes *et al.*, 2001; Egan *et al.*, 2002; Kolterman *et al.*, 2003; Buse *et al.*, 2004). In addition, GLP also suppresses inappropriately high glucagon secretion in a glucose-dependent manner (Kolterman *et al.*, 2003; 2005; Buse *et al.*, 2004), slows gastric emptying (Kolterman *et al.*, 2005), reduces food intake, which in turn promotes weight loss (Szayna *et al.*, 2000; Edwards *et al.*, 2001) and promotes

$\beta$ -cell proliferation and islet neogenesis in diabetic animals (Farilla *et al.*, 2002). Exenatide, the active ingredient of Byetta®, is a synthetic version of the 39-amino-acid peptide originally isolated from the saliva of the gila monster lizard, with 53% amino acid sequences overlapping with GLP-1 (Eng *et al.*, 1992). Exenatide acts as a potent agonist of GLP-1 receptors (Montrose-Rafizadeh *et al.*, 1997) and shows very similar glucoregulatory activities to GLP-1 (DeFronzo *et al.*, 2005; Holst, 2007). As a first-in-class incretin mimetic agent, exenatide has been widely used to improve glycaemic control in type 2 diabetes mellitus patients featured by less hypoglycaemia and progressive weight loss (DeFronzo *et al.*, 2005; Triplitt and DeFronzo, 2006).

However, exenatide exhibits a short plasma half-life of 1.5–4 h and clinical effects lasting for up to 8 h (Parkes *et al.*, 2001; Kolterman *et al.*, 2005) mainly due to its fast kidney excretion (Copley *et al.*, 2006), and requires s.c. injection twice daily for the treatment (Egan *et al.*, 2003; DeFronzo *et al.*, 2005). This has led to main drawbacks of less than optimal clinical compliance (medication possession ratio of 68%) and poorer quality of life (Fabunmi *et al.*, 2009). Reduction of the required frequency of s.c. injections is one way that would significantly enhance compliance. It has been reported that continuous exenatide therapy reached compliance of 100% (Cuddihy *et al.*, 2010). Many drug delivery technologies have been developed to improve exenatide's pharmacokinetic profiles. A once a week long-acting release version of exenatide employing microsphere technology has been demonstrated to be effective for the treatment of type 2 diabetes (Kim *et al.*, 2007). Meanwhile, the exclusive disposition of exenatide via kidney secretion (Copley *et al.*, 2006) offers a unique opportunity for site-specific PEGylation technology to improve exenatide's short plasma duration and poor systemic exposure possibly without reducing its activity.

PEGylation, the covalent attachment of polyethylene glycol (PEG) to targeted drug molecules, has become one of the most mature, valid and widely used drug delivery technologies ever developed. It has generated several blockbuster protein drugs with markedly improved therapeutic properties mainly by extending their plasma lifetimes compared with their corresponding unmodified predecessor molecules (Bailon *et al.*, 2001; Fishburn, 2008; Bailon and Won, 2009). Rational design of a PEGylated exenatide analogue involves several considerations. In order to be able to perform site-specific PEGylation with a maleimide method, exenatide analogues need to be engineered to have a cysteine residue providing a free thiol group, so that a specific C-terminal permanent amide conjugation with maleimide PEGs can be accomplished (Bailon and Won, 2009). It is generally believed that the intact amino acid sequence from 1 through 8 (H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser) of the N-terminal is essential for exenatide to bind GLP-1 receptors and maintain its biological activity, such as stimulation of intracellular cAMP (Lopez de Maturana *et al.*, 2003; Mann *et al.*, 2007). Therefore, the added cysteine should be placed towards the C-terminus site as far as possible from the active site.

Application of random PEGylation usually causes an exponential reduction in binding affinity and bioactivity due to the attachment of PEGs (as little as 4 kDa) near the active site and subsequent steric hindrance of target molecule binding (Bowen *et al.*, 1999; Bailon *et al.*, 2001; Bailon and

Won, 2009). In order to minimize the loss of bioactivity, site-specific PEGylation technologies have emerged using a variety of techniques such as the protein mutation method (Yamamoto *et al.*, 2003; Rosendahl *et al.*, 2005), transglutaminase method (Fontana *et al.*, 2008) and maleimide method (Manjula *et al.*, 2003; Bailon and Won, 2009), which also make the manufacturing process simple and products homogeneous (Bailon and Won, 2009; French *et al.*, 2009). Site-specific PEGylation to proteins of large molecular size, particularly antibody fragments, usually results in no loss of activity up to a PEG50kDa (Yamamoto *et al.*, 2003; Bailon and Won, 2009). However, preserving activity through this type of site-specific PEGylation may not be valid for small-to-medium size peptides such as exenatide due to the short distances between the active site and the conjugation site relative to the molecular size of PEGs.

On the other hand, PEGylation always results in higher plasma levels of PEGylated protein drugs, due both to increased actual and apparent molecular size resulting from the linear structure and hydrophilicity of PEGs (Caliceti and Veronese, 2003; Youn *et al.*, 2006; Bailon and Won, 2009). The *in vivo* efficacy of PEGylated proteins or peptides would be the net effects from PEG-affected activity and prolonged activity produced by extended plasma lifetime (Fishburn, 2008; Bailon and Won, 2009). It is a challenge to optimally select a PEG size based on a balance between the activity and plasma duration by PK/PD modelling. Therefore, the PEG molecular mass-dependency of PEGylation on activities and pharmacokinetics of exenatide analogues needed to be systematically evaluated.

The present study first compared activities of exenatide and PB-105 (ExC39) and PB-102 (ExC35), cysteine-replacement analogues of exenatide at the position 39 and 35, respectively, as well as PB-111 (ExY40), the PB-105 analogue with tyrosine added at the position of 39, on intracellular cAMP stimulation, acute glucoregulatory activity and pharmacokinetic profile. Based on the preservation of its activities, we selected PB-105 as the active intermediate for site-specific PEGylation. The study further investigated PEG mass-dependency of site-specific PEGylation on PB-105's *in vitro* and *in vivo* activities as well as its pharmacokinetic profile. Finally, the study selected PB-106 (ExC39PEG20kDa) for further study based on its favourable combination of activity and plasma duration, and showed it to be twofold to fivefold more efficacious than exenatide in acute glucoregulation in normal and diabetic mice. Preliminary results were presented at the 10th Chinese National Pharmacology Conference (Gong *et al.*, 2009).

## Methods

### Chemicals

Streptozotocin (STZ) was purchased from Sigma (St Louis, MO, USA). Exenatide and its analogues were synthesized by Kaijie Bio-Pharmaceutical Co. (Chengdu, China) using a solid phase method by a multiple peptide synthesizer where an F-moc strategy was employed. As presented in Figure 1, PB-102 (ExC35) and PB-105 (ExC39) are analogues of exenatide formed by replacing alanine and serine with cys-

<b>Exenatide:</b>	H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-A-P-P-P-S-NH <sub>2</sub>
<b>PB-102:</b>	H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-C-P-P-P-S-NH <sub>2</sub>
<b>PB-105:</b>	H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-A-P-P-P-C-NH <sub>2</sub>
<b>PB-111:</b>	H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-A-P-P-P-C-Y-NH <sub>2</sub>

## Figure 1

Amino acid sequences of exenatide and its analogues, PB-102 (ExC35), PB-105 (ExC39) and PB-111 (ExY40).

teine at positions of 35 and 39, respectively, while PB-111 (ExY40) is a PB-105 analogue formed by addition of tyrosine at the position 39. Peptides were characterized by high-performance liquid chromatography and mass spectrometry, as well as sequence analysis in the case of PB-105, with all purities of more than 98%. PEGylated exenatide analogues were manufactured by PegBio Co. (Suzhou, China) employing site-specific PEGylation technology. PB-110 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC39PEG40kDa) were produced by site-specific conjugation of PB-105 linked with a free thiol group provided by the replaced cysteine at the C-terminal with maleimide-derivatized PEG of different molecular masses, that is, 5, 20, 30 and 40 kDa respectively. The purity of all PEGylated peptides was greater than 95% (UV detection). All test substances were dissolved in normal saline except for STZ, which was freshly dissolved in sodium citrate buffer (pH 4.5).

## Intracellular cAMP assay

The pheochromocytoma PC12 cells (obtained from American Type Culture Collection, Rockville, MD, USA) expressing GLP-1 receptors (Perry *et al.*, 2002) were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in a humidified 5% CO<sub>2</sub> air incubator. PC12 cells were plated 48 h prior to the experiment on 24-cell plates at a density of 10<sup>5</sup> cells·mL<sup>-1</sup>. On the day of the experiment when PC12 cells reached 60–70% confluency, the plates were washed twice with PBS buffer and blocked by adding 1 mL of 1% BSA in PBS, then incubated with test compounds and 3-isobutyl-1-methylxanthine (100 µM) for 30 min. The intracellular cAMP was extracted by the addition of 500 µL of 0.1 M hydrochloric acid followed by sonication for 15 s. Intracellular cAMP was determined by using a cAMP ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) by a Microplate Reader (Multiskan MK3, Thermo Labsystems, Vantaa, Finland) while the protein content was quantified by using the BCA method.

## Animals and surgical procedures

Adult male Swiss mice for blood sugar studies, weighing 20–30 g, and adult male Sprague-Dawley rats for pharmacokinetics studies, weighing 250–300 g, were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Following shipment, mice and rats were housed for at least 3 days allowing acclimatization prior to experimental studies. The animals were housed at 23 ± 1°C, and were given access to food and water *ad libitum* unless otherwise noted. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy.

For the pharmacokinetic study, rats were anaesthetized with an i.p. injection of chloral hydrate (300 mg·kg<sup>-1</sup>). Cannulations of the right femoral vein for i.v. injection and of the right femoral artery for sampling were performed with a PE-50 polyethylene cannula (BD Biosciences, San Jose, CA, USA). Cannulas were flushed with 0.2 mL of heparin-treated saline (200 IU·mL<sup>-1</sup>). Experiments were started 16 h after cannulation while animals were fully conscious in home cages, with free access to food and water.

## Blood glucose measurement in normal and diabetics animals

Blood glucose levels were measured by using a glucose oxidase method by OneTouch® Ultra® 2 Blood Glucose Meter (Johnson & Johnson Company, Shanghai, China). Blood samples were diluted in normal saline at a ratio of 3:4 for further measurements in case the blood glucose concentration was higher than 33.3 mmol·L<sup>-1</sup> in some diabetic mice. Diabetes was induced by s.c. injection of STZ at a dose of 120 mg·kg<sup>-1</sup> in mice deprived of food for 12 h. These mice developed diabetes 3 days after STZ injection and those with blood glucose readings higher than 16.7 mM during the 3 day measurements were used in the study.

## Pharmacokinetic and measurement of plasma exenatide analogues by ELISA method

Blood samples (200 µL) were taken (the same volume of heparin-treated saline was flushed back) at different time points before and after injections of exenatide analogues, initially via an indwelling cannula up to 24 h and subsequently from gently squeezing of the tail-tip. The blood samples were centrifuged immediately and stored at -20°C until analysis. Plasma concentrations of exenatide and its analogues were determined using a commercially available enzyme-linked immunosorbent assay kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) that had been validated for determination of exenatide over the concentration range of 0.06–0.68 ng·mL<sup>-1</sup>. The cross-activity of the assay included exenatide, exendin-3(9–39)NH<sub>2</sub> and exendin-4(3–39) (100%), but not human and rat GLP-1(7–36)NH<sub>2</sub>, GLP-1(7–37) and GLP-2; glucagon; human, rat and mouse oxyntomodulin (0%). The activity signal was measured with a Microplate Reader and concentrations of samples were determined by comparison with a calibration curve run at the same time. The linear standard curve (0.1–1 ng·mL<sup>-1</sup>) of exenatide was exactly the same as PB-105 (ExC39), indicating that the replacement of alanine with cysteine at the position of 39 did not affect the immunogenicity of exenatide detected by this assay and the exenatide ELISA kit could be used for detection of PB-105. Furthermore, a slight, insignificant right-shift of

the standard curve of exenatide by PEGylated PB-105 analogues also indicated that site-specific PEGylation had negligible effects on the immunogenicity of exenatide assayed in a PEG mass-dependent manner and this kit was suitable for determination of plasma concentrations of PB-110 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC39PEG40kDa). The intra-assay coefficient of variation for all experiments was  $7.2 \pm 0.4\%$  and inter-assay coefficient of variation for all experiments was  $8.7 \pm 0.9\%$ .

### Data calculations and statistical analysis

Sigmoidal concentration (dose)–response curve analysis was performed to calculate four-parameter logistic regression, including the median effective concentration (dose) ( $EC_{50}$  or  $ED_{50}$ ) and maximum effective dose ( $E_{max}$ ), by using the v5.0 GraphPad Prism Program (GraphPad Software Inc., San Diego, CA, USA). Biological half recovery time ( $t_{1/2}$ ) was calculated by performance of linear regression of the time course curves. Pharmacokinetic parameters were also calculated according to the non-compartmental model using Kinetica Version 5.0 (Thermo Scientific, Inc., Two Rivers, WI, USA). Area under time course curve (AUC) or area above lowered blood glucose versus time course curve (AAC) was calculated from the actual data or extrapolated by  $AAC = 1/2 \times [(2 \times \text{biological half-life value} + \text{peak time value}) \times \text{peak effect value}]$  as noted.

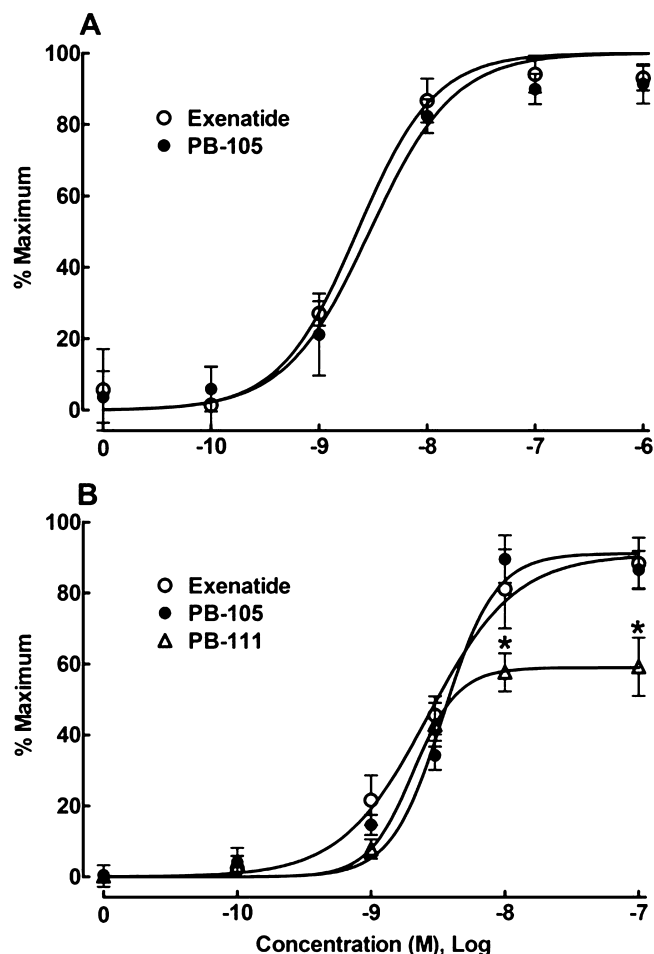
All data are expressed as means  $\pm$  SEM or 95% confidence intervals. Data were analysed for statistical differences by a two-way ANOVA followed by *post hoc* Student-Newman-Keuls test. A *P*-value less than 0.05 was taken to indicate a statistically significant difference between data.

## Results

### Bioactivities of exenatide and its mutant analogues

**Effects of exenatide and its analogues on stimulation of intracellular cAMP in PC12 cells.** The stimulant effect of PB-105 (ExC39), an exenatide analogue with a cysteine substituted at the C-terminus, on intracellular cAMP production in PC12 cells was compared with that of exenatide. After incubation for 30 min, exenatide at varying concentrations ( $10^{-10}$ – $10^{-6}$  M) stimulated cAMP production in a concentration-dependent manner, with an  $E_{max}$  of  $129.4 \pm 6.8$  pmol·mg $^{-1}$  protein and an  $EC_{50}$  of 2.3 nM (95% confidence intervals: 1.3–3.8 nM). PB-105 produced the same concentration-dependent production in cAMP, with an  $E_{max}$  of  $133.2 \pm 7.2$  pmol·mg $^{-1}$  protein and an  $EC_{50}$  of 2.9 nM (95% confidence intervals: 1.7–5.0 nM) (Figure 2A).

PB-111 (ExY40), an analogue of PB-105 with a tyrosine added after position 39 (i.e. position 40), was also compared with PB-105 and exenatide. PB-111 caused a concentration-dependent increase in intracellular cAMP and exhibited the same potency ( $EC_{50}$  = 2.1 nM, 95% confidence intervals: 1.5–2.9 nM) as exenatide (2.7 nM, 95% confidence intervals: 1.7–4.2 nM) or PB-105 (3.5 nM, 95% confidence intervals: 2.7–4.5 nM), but its maximal effect was significantly lower than those of either PB-105 or exenatide by 35.0% ( $P < 0.05$  by ANOVA) (Figure 2B).

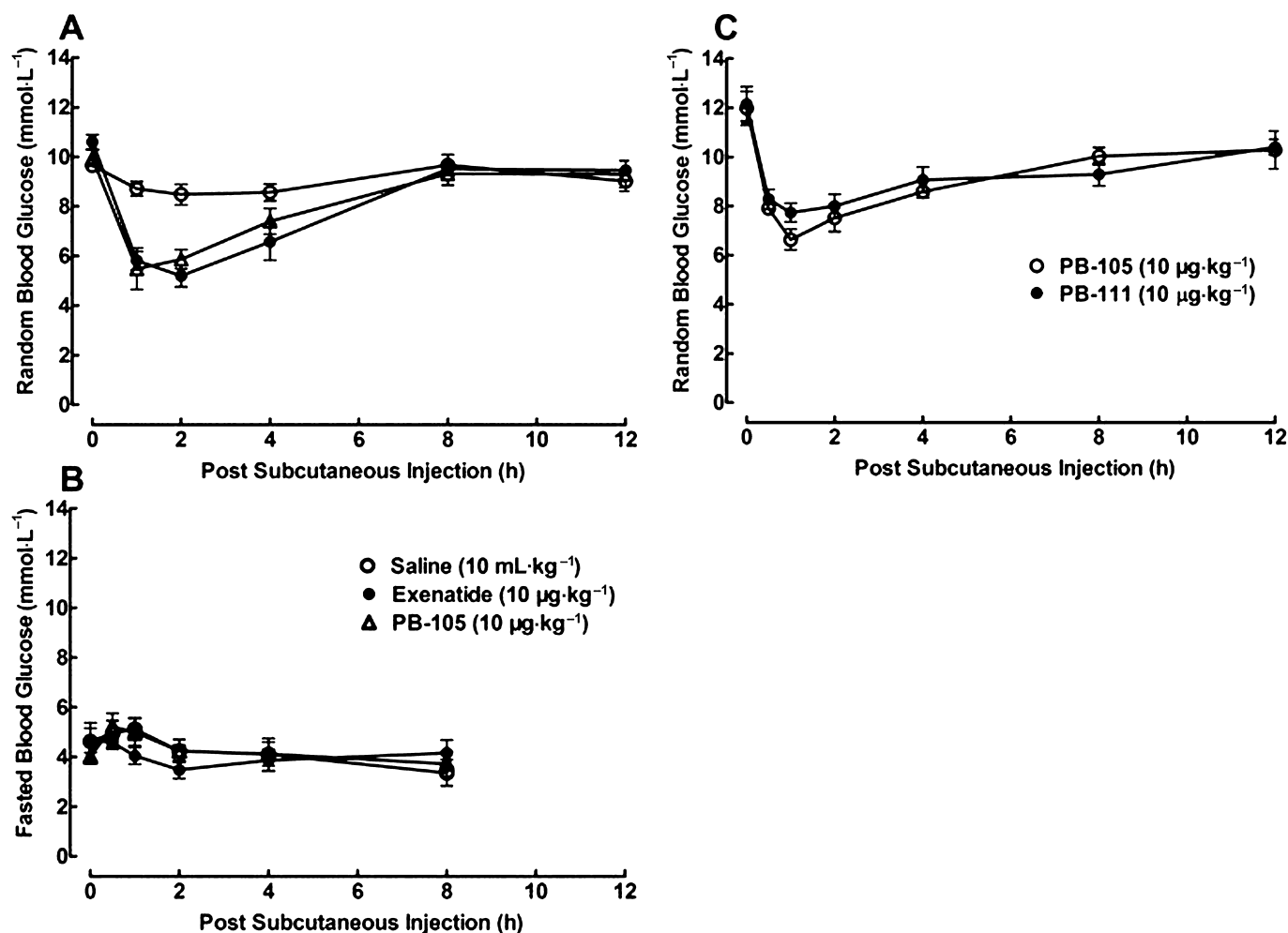


**Figure 2**

Comparisons of concentration–response curves for PB-105 (ExC39) with exenatide (A) and PB-111 (ExY40) (B) on stimulation of intracellular cAMP in PC12 cells. Concentration–response analysis was best fitted by a non-linear least-squares method. All the readings are means  $\pm$  SEM of triplicate results, repeated two to three times. \*Denotes statistically significant difference compared with either exenatide treatment or PB-105 treatment ( $P < 0.05$  by two-way ANOVA followed by Student-Newman-Keuls test).

**Effects of exenatide and its analogues on random and fasted blood glucose levels in normal mice.** Each of three groups of Swiss mice ( $n = 6$  in each group) received s.c. injections of normal saline ( $10 \text{ mL} \cdot \text{kg}^{-1}$ ), PB-105 (ExC39,  $10 \mu\text{g} \cdot \text{kg}^{-1}$ ) or exenatide ( $10 \mu\text{g} \cdot \text{kg}^{-1}$ ). During random blood glucose measurement, mice were allowed free access to food and water. Random blood glucose (representing postprandial sugar) values in saline control mice were in the range of 9.1–11.9 mM and remained stable during the observation period. Both PB-105 and exenatide reduced the random blood glucose levels with a comparable time-dependency, the peak effect occurred after approximately 1 h and the recovery time was approximately 8 h after injection (Figure 3A). The biological half recovery life ( $t_{1/2}$ ) values of PB-105 and exenatide were  $4.4 \pm 0.2$  h and  $4.7 \pm 0.2$  h, and the decreases were  $44.1 \pm 8.9\%$  and  $45.1 \pm 3.4\%$  of initial values. The glucoregulatory activity of exenatide was consistent with previous reports (Young *et al.*,





**Figure 3**

Time courses for the effects of exenatide, PB-105 (ExC39) and PB-111 (ExY40) on random and fasted blood glucose in normal Swiss mice. Data are presented as means  $\pm$  SEM. (A) Three groups of non-fasted mice received single bolus s.c. injections of normal saline (10 mL·kg<sup>-1</sup>), PB-105 (10 µg·kg<sup>-1</sup>) or exenatide (10 µg·kg<sup>-1</sup>);  $n = 8$  in each group. (B) Three groups of fasted (over 16 h) mice received s.c. bolus injections of normal saline (10 mL·kg<sup>-1</sup>), exenatide (10 µg·kg<sup>-1</sup>) or PB-105 (10 µg·kg<sup>-1</sup>);  $n = 5$  in each group. (C) Two groups of non-fasted mice received single bolus s.c. injections of PB-105 (10 µg·kg<sup>-1</sup>) and PB-111 (10 µg·kg<sup>-1</sup>) (dosing volume of 10 mL·kg<sup>-1</sup>);  $n = 6$  in each group.

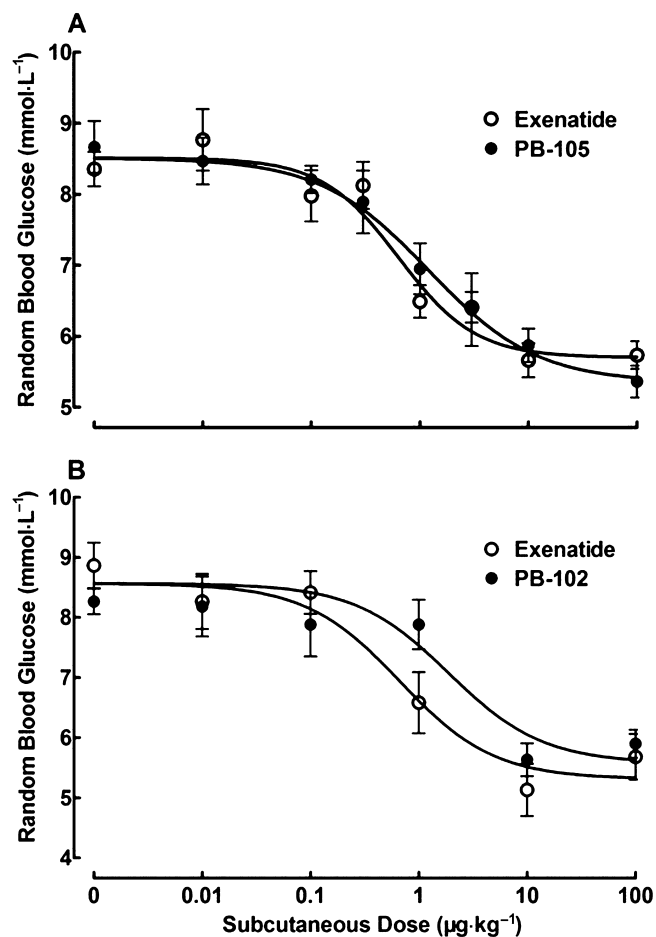
1999; Hargrove *et al.*, 2007). On the other hand, each of the three groups of mice deprived of food for 16 h but with free access to water (fasted groups;  $n = 5$  for each group) also received s.c. injections of saline, exenatide (10 µg·kg<sup>-1</sup>) or PB-105 (10 µg·kg<sup>-1</sup>). As shown in Figure 3B, blood glucose levels in control mice deprived of food dropped to 3.2–7.3 mM and remained stable during the observation period. Neither exenatide nor PB-105 further lowered fasted blood glucose levels at any time point observed.

The time course for the ability of PB-111 (ExY40) to reduce random blood glucose was also compared with PB-105. Each of two groups of non-fasted mice ( $n = 6$  in each group) received s.c. injections of PB-105 (10 µg·kg<sup>-1</sup>) or PB-111 (10 µg·kg<sup>-1</sup>). Both PB-105 and PB-111 reduced blood sugar with a comparable time-dependency; peak time at approximately 1 h and recovery time approximately 8 h after injection (Figure 3C). The  $t_{1/2}$  values of PB-105 and PB-111 were  $4.6 \pm 0.5$  h and  $6.0 \pm 0.8$  h, and the peak effect of

PB-111 ( $36.4 \pm 3.1\%$  of initial values) was slightly and statistically insignificantly smaller than that of PB-105 ( $44.7 \pm 3.6\%$  of initial value) by 18.6%.

Dose-response curves for the effects of PB-105 (ExC39) and exenatide on random blood glucose were determined for normal non-fasted mice. Sixteen groups of mice ( $n = 18$  in each group) received s.c. injections of saline or PB-105 or exenatide at doses of 0.01–100 µg·kg<sup>-1</sup> and the peak blood sugar values were obtained 1 h after injection. Both exenatide and PB-105 caused a dose-dependent reduction in random blood glucose (Figure 4A), with roughly the same maximal reductions of 33.3% and 37.5% of initial values, and ED<sub>50</sub> values of 0.6 µg·kg<sup>-1</sup> (95% confidence intervals: 0.4–1.2 µg·kg<sup>-1</sup>) and 1.2 µg·kg<sup>-1</sup> (95% confidence intervals: 0.5–3.2 µg·kg<sup>-1</sup>).

The dose-response curve for PB-102 (ExC35), an exenatide analogue with a cysteine substituted at the position 35, was also compared with that of exenatide. Twelve groups

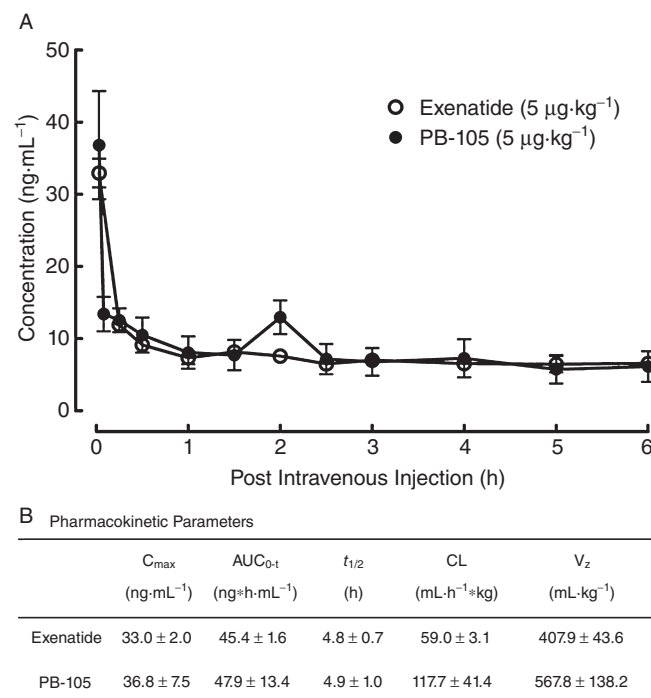


**Figure 4**

Dose-dependent comparisons of exenatide with PB-105 (ExC39) (A) and PB-102 (ExC35) (B) on random blood glucose in normal Swiss mice. Data are presented as means  $\pm$  SEM. (A) Sixteen groups of non-fasted mice received a single bolus s.c. injection of normal saline or PB-105 or exenatide at the doses indicated. The peak blood sugar values were obtained 1 h after injection;  $n = 18$  in each group. (B) Twelve groups of non-fasted mice received single bolus s.c. injections of normal saline, exenatide or PB-102 as indicated. The peak blood sugar values were obtained 1 h after injection;  $n = 6$  in each group.

of non-fasted mice ( $n = 6$  in each group) received s.c. injections of saline, exenatide or PB-102 at doses of 0.01–100  $\mu\text{g}\cdot\text{kg}^{-1}$  and the peak blood sugar values were obtained at 1 h after injection. Both exenatide and PB-102 produced a dose-dependent reduction of random blood sugar (Figure 4B), with maximal effects of 37.9% and 34.6% of initial values. PB-102 was less potent (but not significantly) than exenatide, with  $\text{ED}_{50}$  values of 0.7  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 0.2–2.1  $\mu\text{g}\cdot\text{kg}^{-1}$ ) and 2.0  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 0.6–6.5  $\mu\text{g}\cdot\text{kg}^{-1}$ ).

**Pharmacokinetics of exenatide and PB-105 (ExC39) in rats.** Two groups of cannulated rats received i.v. injections of exenatide or PB-105 (ExC39) at a dose of 5  $\mu\text{g}\cdot\text{mL}^{-1}\cdot\text{kg}^{-1}$ . After the i.v. injection, both plasma exenatide and PB-105 first rapidly decreased in an exponential fashion in the distribution phase



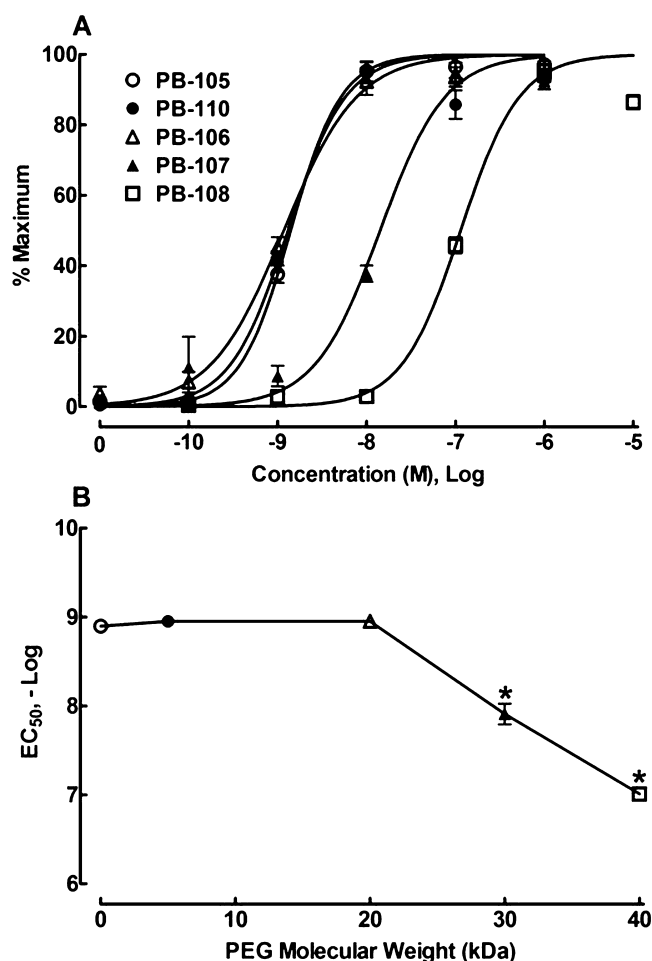
**Figure 5**

Pharmacokinetic profiles (A) and pharmacokinetic parameters (B) of exenatide and PB-105 (ExC39) in normal Sprague-Dawley rats. Two groups of cannulated rats received i.v. injections of exenatide or PB-105 at a dose of 5  $\mu\text{g}\cdot\text{mL}^{-1}\cdot\text{kg}^{-1}$ . Blood samples (200  $\mu\text{L}$ ) were taken (replaced by the same volume of heparin-treated saline) at different time points as indicated. Data are presented as means  $\pm$  SEM;  $n = 3$  in each group. Pharmacokinetic parameters were calculated by using a non-compartment model.

followed by a slow-decay elimination phase (Figure 5A). Pharmacokinetic parameters were calculated using the non-compartment model. Exenatide and PB-105 had elimination phase half-life values of  $4.8 \pm 0.7$  and  $4.9 \pm 1.0$  h respectively. Other pharmacokinetic parameters are also listed in Figure 5B.

### Effects of PB-105 (ExC39) and its PEGylated analogues on the stimulation of intracellular cAMP in PC12 cells

Concentration–response curves for the stimulant effects of PB-105 (ExC39) and its PEGylated analogues with different molecular weights on intracellular cAMP were obtained in PC12 cells. As shown in Figure 6A, PB-105 increased intracellular cAMP in a concentration-dependent manner, with an  $\text{EC}_{50}$  of 1.4 nM (95% confidence intervals: 1.2–1.6 nM) and  $\text{E}_{\text{max}}$  of  $103.9 \pm 1.5$  pmol $\cdot\text{mg}^{-1}$  protein, consistent with the above results. PEGylation with PEGs of up to at least 40 kDa did not affect PB-105's maximal production of intracellular cAMP. However, PEGylation shifted the concentration–response curve of PB-105 to the right in a parallel, PEG mass-dependent manner but with an inflexion point of at least PEF20kDa.  $\text{EC}_{50}$  values of PB-110 (ExC39PEG5kDa), PB-106



**Figure 6**

Concentration–response curves for the effect of PB-105 (ExC39) and its PEGylated analogues on intracellular cAMP in PC12 cells (A) and the relationship between PEG mass (kDa) and potencies of PB-105 and its PEGylated analogues (B). PEGylated analogues conjugated with different molecular masses of PEGs: PB-110 (ExC39PEG5kDa) with PEG5kDa, PB-106 (ExC39PEG20kDa) with PEG20kDa, PB-107 (ExC39PEG30kDa) with PEG30kDa and PB-108 (ExC39PEG40kDa) with PEG40kDa. Concentration–response analysis was best fitted by a non-linear least-squares method. All the readings are means  $\pm$  SEM of triplicate results, repeated two to three times. \*Denotes statistically significant difference compared with PB-105 treatment ( $P < 0.05$  by a two-way ANOVA followed by *post hoc* Student-Newman-Keuls test).

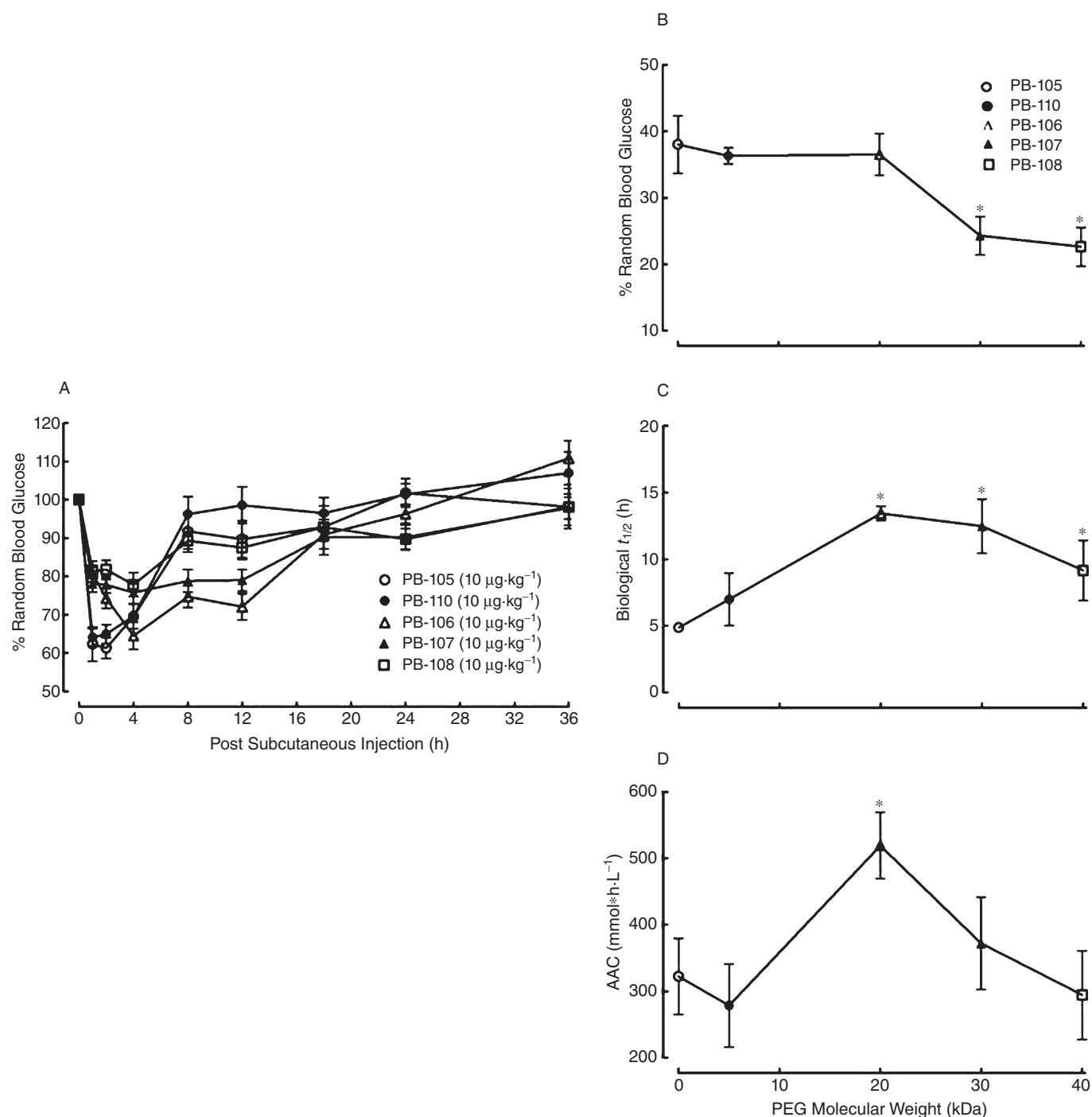
(ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC39PEG40kDa) were 1.3 nM (95% confidence intervals: 1.0–1.8 nM), 1.2 nM (95% confidence intervals: 0.9–1.5 nM), 14.2 nM (95% confidence intervals: 9.9–20.4 nM) and 114.1 nM (95% confidence intervals: 89.7–145.2 nM). The relationship between their PEG mass and log EC<sub>50</sub> values is exhibited in Figure 6B and PEG 20 kDa (at least) was shown to be an inflexion point whereas PB-110 and PB-106 did not affect PB-105's potency, while PB-107 and PB-108 displaced PB-105's potency by approximately 10- and 100-fold respectively.

### Effects of PB-105 (ExC39) and its PEGylated analogues on random blood glucose in normal and diabetic mice

In order to compare the biological durations of PB-105 (ExC39) and its PEGylated analogues, five groups of non-fasted normal mice ( $n = 12$  in each group except for PB-110 group where  $n = 6$ ) received s.c. injections of PB-105, PB-110 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) or PB-108 (ExC39PEG40kDa) at a dose of 10  $\mu\text{g}\cdot\text{kg}^{-1}$ . As shown in Figure 7A, PB-105 produced a reversible reduction in blood glucose the same as shown in Figure 3A, with a peak of  $38.0 \pm 4.3\%$  of initial values 1 h after injection and biological  $t_{1/2}$  of  $4.9 \pm 0.1$  h. PEGylation reduced the peak response to PB-105, slowed and prolonged biological duration of PB-105 in a PEG mass-dependent fashion but also had an inflexion point of at least 20 kDa of PEG. The peak values of PB-110, PB-106, PB-107 and PB-108 were  $36.3 \pm 1.2\%$ ,  $36.5 \pm 3.2\%$ ,  $24.3 \pm 2.8\%$  and  $22.6 \pm 2.9\%$  of initial values with a peak time of approximately 1 h (PB-110) and 4 h (PB-106, PB-107 and PB-108); biological  $t_{1/2}$  values were  $7.0 \pm 2.0$ ,  $13.4 \pm 0.5$ ,  $12.5 \pm 2.0$ ,  $10.8 \pm 2.0$  and  $9.2 \pm 2.2$  h. The relationship between PEG mass and the peak reduction in random blood sugar induced by PEGylated PB-105 analogues as well as biological duration is shown in Figure 7B and C, indicating that at least PEG 20 kDa was an inflexion point where PB-106 prolonged PB-105's biological duration most without reducing its glucoregulatory activity. To evaluate the overall effect on random blood glucose, the AAC was used. The AAC value for PB-105 was  $322.3 \pm 57.4$   $\text{mmol}\cdot\text{h}\cdot\text{L}^{-1}$ , which was significantly increased by PB-106 by 61.1%, but not by PB-110, PB-107 or PB-108 (Figure 7D).

Dose–response curves for the effects of PB-105 (ExC39) and PB-106 (ExC39PEG20kDa) on random blood sugar were further compared. Thirteen groups of normal non-fasted mice ( $n = 6$  in each group) each received s.c. injections of normal saline, PB-105 or PB-106 at doses ranging from 0.1 to 30  $\mu\text{g}\cdot\text{kg}^{-1}$ . Blood glucose levels were assessed 1 h (saline group and PB-105 groups) or 4 h (PB-106 groups) after administration. Both peptides reduced blood sugar in a dose-dependent manner (Figure 8A). Maximal falls in blood glucose with each peptide approached 33.5% and 38.4% of initial values. ED<sub>50</sub> values of PB-105 and PB-106 were 1.1  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 0.5–2.6  $\mu\text{g}\cdot\text{kg}^{-1}$ ) and 3.0  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 1.2–9.3  $\mu\text{g}\cdot\text{kg}^{-1}$ ). AACs were extrapolated by the formula of  $\text{AAC} = 1/2 \times [(2 \times \text{biological half-life value} + \text{peak time value}) \times \text{peak effect value}]$ , where mean biological  $t_{1/2}$  values of 4.9 h for PB-105 and 13.4 h for PB-106, respectively, were derived from Figure 7A, and were selected to integrate biological durations and magnitudes of reduction. As shown in Figure 8B, PB-105 lowered blood sugar in a dose-dependent manner, with an ED<sub>50</sub> value of 1.8  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 1.1–2.8  $\mu\text{g}\cdot\text{kg}^{-1}$ ) and an AAC E<sub>max</sub> of  $11.6 \pm 0.9$  ( $\text{mmol}\cdot\text{h}\cdot\text{L}^{-1}$ ). PB-106 also dose-dependently lowered blood sugar with an ED<sub>50</sub> value of 4.5  $\mu\text{g}\cdot\text{kg}^{-1}$  (95% confidence intervals: 1.9–10.7  $\mu\text{g}\cdot\text{kg}^{-1}$ ) and an AAC E<sub>max</sub> of  $37.8 \pm 7.1$  ( $\text{mmol}\cdot\text{h}\cdot\text{L}^{-1}$ ), which was 3.2-fold that of PB-105's potency ( $P < 0.05$ ).

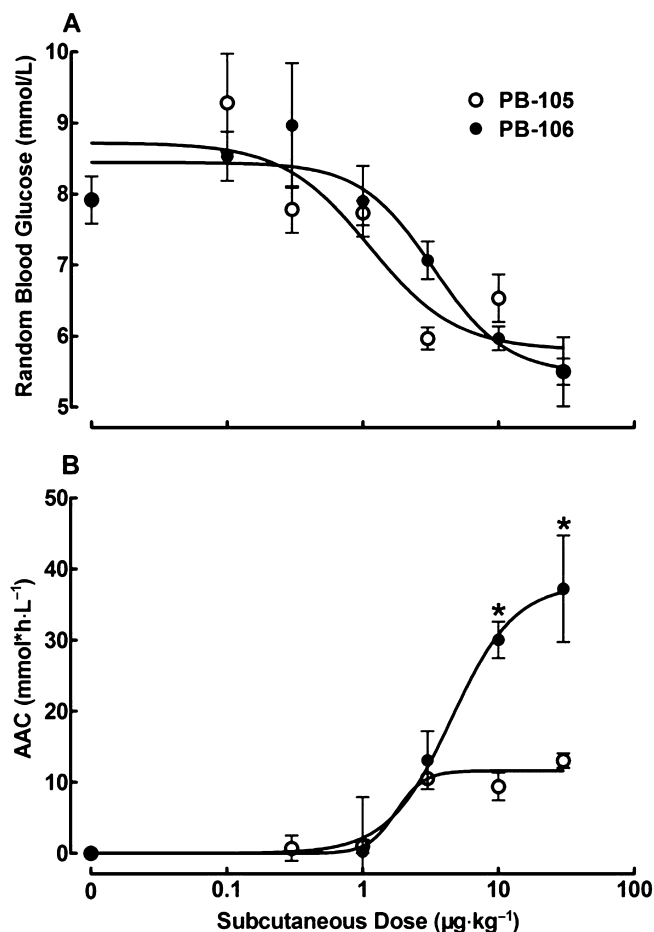
The lowering of blood sugar by exenatide, PB-105 (ExC39) and PB-106 (ExC39PEG23kDa) were further tested in diabetic



## Figure 7

Time courses for the effects of PB-105 (ExC39) and its PEGylated analogues on random blood sugar levels in normal mice (A), and the correlation between PEG molecular weight and the maximal lowering effect ( $E_{\text{max}}$ ) (B), biological half-life ( $t_{1/2}$ ) (C) and area above time course curve (AAC) (D). PEGylated analogues conjugated with different molecular masses of PEGs: PB-110 (ExC39PEG5kDa) with PEG5kDa, PB-106 (ExC39PEG20kDa) with PEG20kDa, PB-107 (ExC39PEG30kDa) with PEG30kDa and PB-108 (ExC39PEG40kDa) with PEG40kDa. Six groups of non-fasted normal mice received bolus s.c. injections of PB-105, PB-110, PB-106, PB-107 or PB-108 at a dose of  $10 \mu\text{g}\cdot\text{kg}^{-1}$  (dosing volume:  $10 \text{ mL}\cdot\text{kg}^{-1}$ ). Data are presented as means  $\pm$  SEM;  $n = 12$  in each group except for the PB-110 group where  $n = 6$ . \*Denotes statistically significant difference compared with PB-105 group ( $P < 0.05$  by a two-way ANOVA followed by *post hoc* Student-Newman-Keuls test).

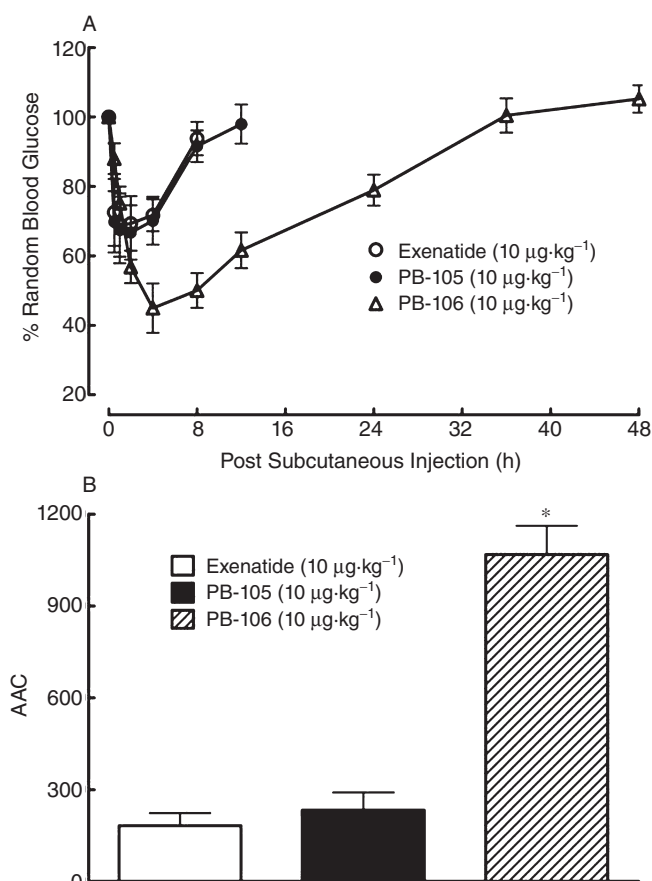




**Figure 8**

Dose–response curves for the effects of PB-105 (ExC39) and PB-106 (ExC39PEG20kDa) on random blood glucose levels (A) and areas above curves (AACs, B) in normal Swiss mice. Thirteen groups of non-fasted mice received bolus s.c. injections of normal saline, PB-105 or PB-106 at the doses indicated. Blood glucose levels were assessed 1 h (normal saline group and PB-105 groups) or 4 h (PB-106 groups) after administration. (B) AACs were selected to integrate biological durations and drop magnitudes and were extrapolated by the formula  $AAC = 1/2 \times [(2 \times \text{biological half-life value} + \text{peak time value}) \times \text{peak effect value}]$ , where the mean biological  $t_{1/2}$  values of 4.9 h for PB-105 and 13.4 h for PB-106, respectively, were derived from (A). Data are presented as means  $\pm$  SEM;  $n = 6$  in each group. \*Denotes statistically significant difference compared with PB-105 group ( $P < 0.05$  by two-way ANOVA followed by Student-Newman-Keuls test).

mice. Swiss mice developed diabetes with random blood sugar ranging from 16.7 to 44.4 mmol·L<sup>-1</sup> 3 days after s.c. injection of STZ (120 mg·kg<sup>-1</sup>). Three groups of STZ-induced non-fasted diabetic mice ( $n = 7$ –11 in each group) each received s.c. injections of exenatide, PB-105 or PB-106 at a dose of 10 μg·kg<sup>-1</sup>. As shown in Figure 9A, exenatide and PB-105 exhibited reversible lowering of blood glucose, with similar peak drops in random blood glucose ( $37.7 \pm 5.7\%$  of initial values for exenatide vs.  $40.4 \pm 8.8\%$  of initial values for PB-105) and biological duration ( $t_{1/2}$  of  $6.1 \pm 0.6$  h for exenatide vs.  $5.6 \pm 0.3$  h for PB-105), close to the results for



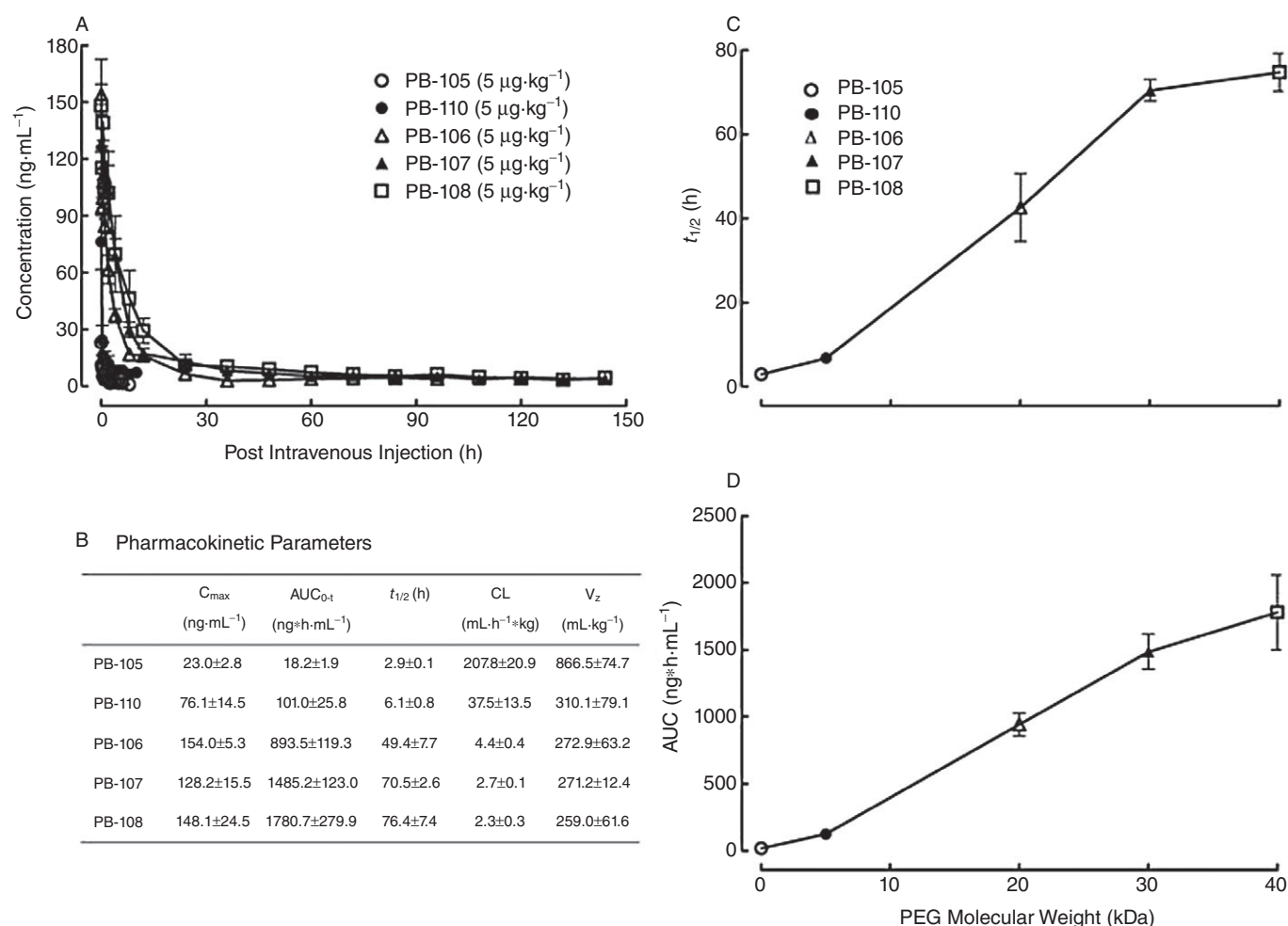
**Figure 9**

Time courses for the effects of exenatide, PB-105 (ExC39) and PB-106 (ExC39PEG20kDa) on random blood glucose levels expressed as fall in magnitude (A) and areas above curves (AACs, B) in streptozotocin (STZ)-induced diabetic mice. These mice developed diabetes with initial random blood sugar ranging from 16.7 to 44.4 mmol·L<sup>-1</sup> 3 days after s.c. injection of STZ (120 mg·kg<sup>-1</sup>). Data are presented as means  $\pm$  SEM;  $n = 7$ –11 in each group. \*Denotes statistically significant difference compared with either exenatide group or PB-105 group ( $P < 0.05$  by ANOVA followed by Student-Newman-Keuls test).

normal mice suggesting the diabetic phenotype did not affect the ability of these peptides to decrease blood glucose. Compared with exenatide or PB-105, PEG-106 produced a larger, but not statistically significant, decrease in random blood sugar of  $55.1 \pm 6.4\%$  and a longer biological duration ( $t_{1/2}$  of  $23.0 \pm 1.3$  h). To integrate the overall glucoregulatory activity with biological duration and magnitude of reductions in blood sugar, the calculated AACs were selected for a comparison. PB-106 produced profound glucoregulatory effects ( $P < 0.05$  vs. PB-105 or exenatide), which were 5.9-fold and 4.6-fold those of exenatide and PB-105 respectively (Figure 9B).

### Pharmacokinetics of PB-105 (ExC39) and its PEGylated analogues in normal rats

Five groups of normal Sprague-Dawley rats ( $n = 3$  in each group) each received bolus i.v. injections of PB-105 (ExC39)



**Figure 10**

Pharmacokinetic profiles (A) and parameters (B) of PB-105 and its PEGylated analogues in normal Sprague-Dawley rats, and the correlation between PEG mass (kDa) and plasma half-life ( $t_{1/2}$ ) (C) and systemic exposure (AUC) (D). PEGylated analogues conjugated with different molecular masses of PEGs: PB-110 (ExC39PEG5kDa) with PEG5kDa, PB-106 (ExC39PEG20kDa) with PEG20kDa, PB-107 (ExC39PEG30kDa) with PEG30kDa and PB-108 (ExC39PEG40kDa) with PEG40kDa. Five groups of cannulated rats received bolus i.v. injections of PB-105 or its PEGylated analogues at a dose of 5 μg·mL<sup>-1</sup>·kg<sup>-1</sup>. Blood samples (200 μL) were taken (replaced by the same volume of heparin-treated saline) at different time points ranging from 0.03 h to 6 h (PB-105), 10 h (PB-110), 96 h (PB-106), 144 h (PB-107) and 168 h (PB-108) post administration. Data are presented as means ± SEM;  $n = 3$  in each group. Pharmacokinetic parameters were calculated by using a non-compartmental model.

or its PEGylated analogues at a dose of 5 μg·mL<sup>-1</sup>·kg<sup>-1</sup>. Figure 10A illustrates plasma concentration profiles of these peptides during scheduled experimental periods. Pharmacokinetic parameters calculated by using a non-compartment model are listed in Figure 10B. Unmodified PB-105 was rapidly removed from the circulation first in an exponential fashion in the distribution phase followed by a slow-decay elimination phase, with an elimination phase  $t_{1/2}$  of  $2.9 \pm 0.1$  h, whereas PEGylated PB-105 analogues were found to have extended plasma lifetimes with increasing PEG molecular weights from 5 to 40 kDa. Specifically, the plasma  $t_{1/2}$  values of PB-110 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC39PEG40kDa) were linearly increased to  $6.1 \pm 0.8$ ,  $49.4 \pm 7.7$ ,  $70.5 \pm 2.6$  and  $76.4 \pm 7.4$  h (Figure 10C). PEGylation also linearly increased the AUC of PB-105 with an increasing

in PEG mass (Figure 10D), whereas the apparent volume of distribution ( $V_z$ ) and systemic clearance (CL) of PB-105 were reduced in a PEG size-dependent fashion.

## Discussion

In contrast to the intact amino acid sequence from 1 through to 8 (H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser) of the N-terminal is required for exenatide to bind GLP-1 receptors and maintain its biological activity (Lopez de Maturana *et al.*, 2003; Mann *et al.*, 2007), its extension of nine amino acid residues (Leu 21-Pro 38), known as the 'Trp cage' of exendin-4, was reported to be negligibly involved in GLP-1 receptor binding (Neidigh *et al.*, 2001; Runge *et al.*, 2007; 2008). Our data support this notion and show that replacement of alanine with cysteine at

position of 35 of exenatide (PB-102, ExC35) slightly, but not significantly, reduced the potency of exenatide.

The importance of the serine residue at position 39 in the binding of the compound to GLP-1 receptors has not been studied, although it was found that deletion of the last three amino acid residues slightly, but significantly, reduced the GLP-1 receptor binding affinity (Doyle *et al.*, 2003). We extensively studied this and found that (i) both PB-105 (ExC39) and exenatide had the same potency and efficacy at increasing the production of intracellular cAMP; (ii) both PB-105 and exenatide displayed the same acute reduction of random blood sugar but not fasting blood sugar in normal and diabetic mice, induced by comparing their biological durations and dose-response curves; and (iii) both peptides also exhibited the same pharmacokinetic profiles, including plasma duration, systemic exposure, volume of distribution and systemic clearance. Thus, substitution of serine with cysteine at position 39 not only preserves the activity of exenatide, but also provides a free thiol group for conjugation. PB-105 was therefore selected as an active intermediate for further site-specific PEGylation. PB-105 would be expected to keep the same or have better glucoregulatory properties in humans, as it was recently reported that the C-terminal region of exenatide is more important for binding to rat GLP-1 receptors than to human GLP-1 receptors (Mann *et al.*, 2010).

Random PEGylation of interferon (Bailon *et al.*, 2001) and of granulocyte colony-stimulating factor (Bowen *et al.*, 1999) has been shown to impair their *in vitro* activity in an exponential manner, starting with as little as 4 kDa of PEGs. The typical 'Exponential-Shift' of bioactivity by random PEGylation usually means the doses needed to produce a particular effect have to be increased, which possibly results in more adverse effects. On the other hand, site-specific PEGylation with proteins of large molecular size, particularly antibody fragments, usually results in no activity loss up to PEG50kDa in a 'No-Shift' manner (Chapman *et al.*, 1999; Chapman, 2002; Bailon and Won, 2009). For example, site-specific PEGylated Fab' with PEG molecule of 40 kDa was shown to preserve full binding activity, in contrast to random PEGylation resulting in 50% loss of binding activity (Chapman *et al.*, 1999; Chapman, 2002).

However, in the present study a new type of 'Inflexion-Shift' by site-specific PEGylation of small peptides was demonstrated. We found that site-specific PEGylation did not reduce either PB-105's intracellular cAMP activity or its acute glucoregulatory activity until PEGs reached at least 20 kDa in molecular mass. The feature of 'Inflexion-Shift' by site-specific PEGylation with permanent (not cleavable) linkage to the PEG molecule is particularly important for small peptides of a few kDa in molecular size such as exenatide, as it is generally believed that cleavable linkages should be used for these peptides or small organic compounds (Bailon and Won, 2009).

Our study showed that PEGylation greatly improved the poor pharmacokinetic profile of exenatide by increasing the elimination phase  $t_{1/2}$  and AUC, as well as reducing  $V_z$  and CL, in a PEG mass-dependent manner. Of the PEGylated PB-105 analogues studied, PB-106 (ExC39PEG20kDa) was found to have a plasma half-life of 43 h, approximately 10-fold higher than exenatide. Increased effective molecular size has two contrary effects on biological activity and metabolic sta-

bility. Thus selection of PEG molecular weight should be based on a balance between both factors, preferably increasing plasma duration and systemic exposure without sacrificing intrinsic biological activity. PB-106, with its increased plasma half-life and preserved *in vitro* activity and *in vivo* glucoregulatory activity (expressed as the drop magnitude compared with initial values) would be expected to be effective in stabilizing postprandial glycaemic challenges.

PB-106 was actually 2.2-fold more effective than PB-105 in postprandial random glucoregulation when the increased plasma duration and drop in magnitude of blood glucose were integrated (expressed as the area above the time course curves). The enhancement of glucoregulation by PEGylation was amplified in diabetic mice, where PB-106 was roughly 3.4- and 4.9-fold more superior to exenatide or PB-105. The excellent efficacy of PB-106 along with its improved pharmacokinetic profile suggests that this exenatide analogue could be useful in the long-term management of diabetes. It is known that long-term treatment by twice daily exenatide is used to successfully manage type 2 diabetic animals and humans leading to a reduction of blood HbA1c (a measurement of prior 3 month blood glucose level) as well as body weight and food intake (which are beneficial effects for diabetes patients) (Greig *et al.*, 1999; Szayna *et al.*, 2000). PB-106's increases in acute glucoregulatory effects in both normal mice (twofold to threefold superior) and diabetic mice (fourfold to fivefold superior) relative to exenatide or PB-105 translated to its long-term effects in GK diabetic rats. This was demonstrated by showing that PB-119 (ExC39PEG23kDa), another PEGylated (PEG23kDa) analogue of PB-105 (ExC39) administered once every 2 days was as equally effective as twice-a-day exenatide or PB-105 in reducing blood HbA1c and body weight as well as food intake in GK diabetic rats (N. Gong *et al.*, unpubl. data).

Our results showed that exenatide and PB-105 produced an acute and reversible glucoregulatory effect, consistent with previous reports (Young *et al.*, 1999; Hargrove *et al.*, 2007). The acute glucoregulatory activity of exenatide and PB-105 observed had the following characteristics: (i) although the maximal reduction in random blood glucose (representing postprandial sugar) was greater in STZ diabetic mice, that were initially hyperglycaemic, than in normal mice with normal blood glucose, the magnitude of the reduction in blood glucose was independent of the initial random blood sugar levels and was roughly 35–45% of initial values, as demonstrated by the same peak effect in normal Swiss mice (the present study) and C57/BL/6 mice (Hargrove *et al.*, 2007), STZ-induced diabetic mice (the present study), db/db diabetic mice (Young *et al.*, 1999; Hargrove *et al.*, 2007) and ob/ob diabetic mice (Young *et al.*, 1999), and in diabetic rhesus monkeys (Young *et al.*, 1999). (ii) This effect is species-specific, as acute administration of exenatide or PB-105 were effective in mice (the present study; Young *et al.*, 1999; Hargrove *et al.*, 2007) and monkeys (Young *et al.*, 1999) but did not reduce random blood sugar levels in normal Wistar rats or GK diabetic rats (N. Gong *et al.*, unpubl. data). Acute but not chronic administration of exenatide has been shown to exhibit a paradoxical increase in glucose in rats (but not in mice) probably through activation of the sympathetic nervous system (Malendowicz *et al.*, 2001; Perez-Tilve *et al.*, 2010). (iii) Neither exenatide nor PB-105 produced an acute

regulatory effect in the fasted mice, similar to previous studies (Nauck *et al.*, 1993; Qualmann *et al.*, 1995; Baggio *et al.*, 2000). This is due to the strict glucose-dependence of the insulinotropic effect of GLP-1 that limits the amount of insulin secreted when high doses of GLP-1 or similarly acting analogues are administered at fasted plasma glucose concentrations, demonstrated in normal or type 2 diabetic animals (Young *et al.*, 1999) and diabetic mellitus humans (Kolterman *et al.*, 2003; Kendall *et al.*, 2005). (iv) Acute glucoregulatory effects (magnitudes) of exenatide and PB-105 can be integrated with their biological durations and expressed as area above the curve, which more precisely predicts their long-term anti-diabetic properties. Thus the mouse acute blood glucose measurement might provide a simple, reliable and predictable assay for reflecting the glucoregulatory nature of the GLP-1 system in normal and diabetic conditions, and might be useful for screening for GLP-1 receptor agonists.

In summary, site-specific PEGylation shifted the concentration-response curve of PB-105 (ExC39) to the right in a parallel, PEG mass-dependent manner but had an inflexion point of at least PEG20kDa. PEGylation also affected acute *in vivo* glucoregulatory activity (the magnitude) in an 'Inflexion-Shift' fashion at PEG20kDa (at least), but linearly increased plasma duration and overall systemic exposure in a PEG mass-dependent manner without an inflexion point. PB-106 (ExC30PEG20kDa) had a plasma  $t_{1/2}$  of 43 h, approximately 10-fold that of PB-105 or exenatide. PB-106 was also found to exhibit much better glucoregulatory activity compared with exenatide or PB-105, twofold to threefold better in normal mice and fourfold to fivefold in diabetic mice. Our results show that site-specific PEGylation of small peptides in permanent amide linkage affects their activity in a new type of 'Inflexion-Shift' fashion, in contrast to the previously demonstrated 'Exponential-Shift' pattern in random PEGylation or the 'No-Shift' pattern in site-specific PEGylation with protein drugs of large molecular mass. PB-106 was designed to be a putative new molecular entity for treating diabetes, with no loss of *in vitro* activity but prolonged plasma duration and consequently markedly improved *in vivo* glucoregulatory activity.

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## Conflict of interest

LJZ, XSL, YHZ and MX are employees of PegBio Co. Ltd. that is developing PEGylated exenatide analogues for the treatment of type 2 diabetes and financially sponsored the study, in part.

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