CHAPTER 6

References


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

Nian Gong1, Al-Niu Ma1, Li-Jie Zhang2, Xiao-Su Luo2, Yin-Hui Zhang2, Michael Xu2 and Yong-Xiang Wang1

1King's Lab, School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China, and 2PegBio Co., Ltd., Suzhou, Jiangsu, China

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 t1/2 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
AUC, 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; t1/2, 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 pericellular cells, α- and β-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; Koltermann et al., 2003; Buse et al., 2004). In addition, GLP also suppresses inappropriate high glucagon secretion in a glucose-dependent manner (Koltermann et al., 2003; 2005; Buse et al., 2004), slows gastric emptying (Koltermann et al., 2005), reduces food intake, which in turn promotes weight loss (Szanya et al., 2000; Edwards et al., 2001) and promotes...
β-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; Koltermann 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 lead to main drawbacks of less than optimal clinical compliance (medication possession ratio of 68%) and poorer quality of life (Sahum 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 (Ballon et al., 2001; Fishburn, 2008; Ballon 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 (Ballon and Won, 2009). It is generally believed that the intact amino acid sequence from 1 through 8 (H-His-Gly-Glu-Gly-Asp-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; Ballon et al., 2001). Ballon 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; Ballon and Won, 2009), which also make the manufacturing process simple and products homogeneous (Ballon 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; Ballon 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; Yoon et al., 2006; Ballon 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; Ballon 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 Kaite 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-
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 PegBioc Co. (Suzhou, China) employing site-specific PEGylation technology. PB-105 (ExC39PEG5kDa), PB-106 (ExC39PEG20kDa), PB-107 (ExC39PEG30kDa) and PB-108 (ExC30PEG40kDa) 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 phaeochromocytoma 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% CO₂ air incubator. PC12 cells were plated 48 h prior to the experiment on 24-well plates at a density of 10⁴ cells/mL⁻¹. 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⁻¹). 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 U·mL⁻¹). 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 diabetic 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⁻¹ in some diabetic mice. Diabetes was induced by s.c. injection of STZ at a dose of 120 mg·kg⁻¹ 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⁻¹. The cross-reactivity of the assay included exendin-3(9–39)NH₂ and exendin-4(3–39) (100%), but not human and rat GLP-1(7–36)NH₂, 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⁻¹) 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 ± 0.4% and inter-assay coefficient of variation for all experiments was 8.7 ± 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) (EC50 or ED50) and maximum effective dose (Emax), by using the v5.0 GraphPad Prism Program (GraphPad Software Inc., San Diego, CA, USA). Biological half recovery time (t1/2) 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 × (t ∫ t biological half-life value + peak time value) × peak effect value as noted.

All data are expressed as means ± 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 (10⁻⁶-10⁻¹⁰ M) stimulated CAMP production in a concentration-dependent manner, with an Emax of 129.4 ± 6.8 pmol·mg⁻¹ protein and an EC50 of 2.3 nM (95% confidence intervals: 1.3-3.8 nM). PB-105 produced the same concentration-dependent production in CAMP, with an Emax of 133.2 ± 7.2 pmol·mg⁻¹ protein and an EC50 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 (EC50 = 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% (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 ± 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 mL·kg⁻¹), PB-105 (ExC39, 10 μg·kg⁻¹) or exenatide (10 μg·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 nM 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 (t1/2) values of PB-105 and exenatide were 4.4 ± 0.2 h and 4.7 ± 0.2 h, and the increases were 44.1 ± 8.9% and 45.1 ± 3.4% of initial values. The glucose regulatory 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 ± SEM. (A) Three groups of non-fasted mice received s.c. injections of normal saline (10 mL kg⁻¹), PB-105 (10 µg kg⁻¹) or exenatide (10 µg kg⁻¹); 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⁻¹), exenatide (10 µg kg⁻¹) or PB-105 (10 µg kg⁻¹); n = 5 in each group. (C) Two groups of non-fasted mice received s.c. injections of PB-105 (10 µg kg⁻¹) and PB-111 (10 µg kg⁻¹) (dosing volume of 10 mL kg⁻¹); 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⁻¹) or PB-105 (10 µg kg⁻¹). 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⁻¹) or PB-111 (10 µg kg⁻¹). 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 t½ values of PB-105 and PB-111 were 4.6 ± 0.3 h and 6.0 ± 0.8 h, and the peak effect of PB-111 (36.4 ± 3.1% of initial values) was slightly and statistically insignificantly smaller than that of PB-105 (44.7 ± 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 0–100 µg kg⁻¹ 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 Emax values of 0.6 µg kg⁻¹ (95% confidence intervals: 0.4–1.2 µg kg⁻¹) and 1.2 µg kg⁻¹ (95% confidence intervals: 0.5–3.2 µg kg⁻¹).

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 ± 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 µg·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 E_max values of 0.7 µg·kg⁻¹ (95% confidence intervals: 0.2–2.1 µg·kg⁻¹) and 2.0 µg·kg⁻¹ (95% confidence intervals: 0.6–6.5 µg·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 µg·mL⁻¹·kg⁻¹. After the i.v. injection, both plasma exenatide and PB-105 first rapidly decreased in an exponential fashion in the distribution phase 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 ± 0.7 and 4.9 ± 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 EC₅₀ of 1.4 nM (95% confidence intervals: 1.2–1.6 nM) and E_max of 103.9 ± 1.5 pmol·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 parallel, PEG mass-dependent manner but with an inflexion point of at least PEG20kDa). EC₅₀ values of PB-110 (ExC39PEG5kDa), PB-106

Table 1: Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>C_max</th>
<th>AUC₂₄</th>
<th>t₁/2</th>
<th>CL</th>
<th>V_d</th>
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<tr>
<td>Exenatide</td>
<td>33.0 ± 2.0</td>
<td>44.4 ± 1.6</td>
<td>4.6 ± 0.7</td>
<td>58.0 ± 3.1</td>
<td>4079 ± 43.6</td>
</tr>
<tr>
<td>PB-105</td>
<td>36.8 ± 7.5</td>
<td>47.9 ± 5.4</td>
<td>4.9 ± 1.0</td>
<td>1177 ± 41.4</td>
<td>5678 ± 138.2</td>
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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 (Exc39PEG3kDa), PB-106 (Exc39PEG20kDa), PB-107 (Exc39PEG30kDa) or PB-108 (Exc39PEG40kDa) at a dose of 10 μg·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 ± 4.3% of initial values 1 h after injection and biological t½ of 4.9 ± 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 ± 1.2%, 36.5 ± 3.2%, 24.3 ± 2.8% and 22.6 ± 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 t½ values were 7.0 ± 2.0, 13.4 ± 0.5, 12.5 ± 2.0, 10.8 ± 2.0 and 9.2 ± 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 were 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 ± 57.4 mmol·h·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 μg·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. ED₅₀ values of PB-105 and PB-106 were 1.1 μg·kg⁻¹ (95% confidence intervals: 0.5–2.6 μg·kg⁻¹) and 3.0 μg·kg⁻¹ (95% confidence intervals: 1.2–9.3 μg·kg⁻¹). AAs were extrapolated by the formula of AAC = \( 1/2 \times (2 \times \text{biological half-life value} + \text{peak time value}) \times \text{peak effect value} \), where mean biological t½ 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 ED₅₀ value of 1.8 μg·kg⁻¹ (95% confidence intervals: 1.1–2.8 μg·kg⁻¹) and an AAC of 11.6 ± 0.9 (mmol·h·L⁻¹). PB-106 also dose-dependently lowered blood sugar with an ED₅₀ value of 4.5 μg·kg⁻¹ (95% confidence intervals: 1.9–10.7 μg·kg⁻¹) and an AAC of 37.8 ± 7.1 (mmol·h·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_{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 (ExC39PEG30kDa) 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 μg kg^{-1} (dosing volume: 10 mL kg^{-1}). Data are presented as means ± 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 2 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 × [(2 × biological half-life value + peak time value) × peak effect value], where the mean biological half-life 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 ± 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).

Figure 9
Time course 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 glucose ranging from 16.7 to 44.4 mmol·L⁻¹ 3 days after s.c. injection of STZ (120 mg·kg⁻¹). Data are presented as means ± 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).

Figure 10
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) 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 ± 6.4% and a longer biological duration (t½ of 23.0 ± 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).
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⁻¹·kg⁻¹. 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.

Figure 10A illustrates plasma concentration profiles of these peptides during scheduled experimental periods. Pharmacokinetic parameters calculated by using a non-compartmental 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 ± 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 ± 0.8, 7.5 ± 0.8, 7.7, 7.4 ± 0.2 and 7.4 ± 0.4 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 ($V_d$) 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-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 (Loepe de Matuana 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 (Neldigh 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 PEylation. PB-105 would be expected to keep the same or have better glycodynamic 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 PEylation of interferon (Bailon et al., 2001) and of granulocyte colony-stimulating factor (Bowen et al., 1999) has 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 PEylation 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 PEylation 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 PEylated Fab’ with PEG molecule of 40 kDa was shown to preserve full binding activity, in contrast to random PEylation 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 PEylation of small peptides was demonstrated. We found that site-specific PEylation did not reduce either PB-105’s intracellular cAMP activity or its acute glycodynamic activity until PEGs reached at least 20 kDa in molecular mass. The feature of ‘inflexion-Shift’ by site-specific PEylation 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 PEylation greatly improved the poor pharmacokinetic profile of exenatide by increasing the elimination phase t1/2 and AUC, as well as reducing Vl and CL, in a PEG mass-dependent manner. Of the PEylated PB-105 analogues studied, PB-106 (ExC39PEG20kDa) was found to have a plasma half-life of 45 h, approximately 10-fold higher than exenatide. Increased effective molecular size has two contrary effects on biological activity and metabolic stability. 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 glycodynamic 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 glucose regulation 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 glycodynamic by PEylation 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; Szanya et al., 2000). PB-106’s increases in acute glycodynamic 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 PEylated (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 glycodynamic effect, consistent with previous reports (Young et al., 1999; Hargrove et al., 2007). The acute glycodynamic 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 C57BL/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-Dive 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 (Koltermann 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 PEylation 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. PEylation 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 PEylation 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 PEylation or the "No-Shift" pattern in site-specific PEylation 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.

Acknowledgements

This work is financially supported by the Mega New Drug Development Program of China (Grant No. 2009ZX09102-257) and a Shanghai Jiao Tong University School of Pharmacy Predoctoral Fellowship to N.G. We thank Dr Qing-Shan Zheng at Shanghai University of Chinese Medicine (Shanghai, China) for discussion on data calculation and statistics and Dr George Milliamich at Airmid Inc. (Redwood City, CA, USA) for editing the manuscript.

Conflict of interest

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

References


Part II

The site-specific PEGylated analog of exendin-4 delayed natural deterioration of glycaemic control in type 2 diabetic Goto-Kakizaki rats

Nian Gong¹, Yan-Chao Wang¹, Hui-Li Wang¹, Micheal Xu² and Yong-Xiang Wang¹, *

¹King’s Lab, School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China, and ²PegBio Co., Suzhou, Jiangsu, China

*Corresponding author: Tel.: +86-21-3420-4763; Email: yxwang@sjtu.edu.cn

Running title: PEGylated exendin-4 on glycaemic control

Abstract

We have previously reported site-specific PEGylation of mutant exenatide with a permanent amide linkage affects its activity in a new type of “Inflexion-Shift” fashion with an inflexion point of at least 20 kDa, which possesses no loss of in vitro activity, prolonged plasma duration and superior, improved in vivo glucoregulatory activity compared with exenatide. Here, we provided a higher molecular weight PEGylated analog with an extended inflexion point of 23 kDa (PB-119). PB-119 showed a full in vitro activity and longer duration of action. For a nearly half exenatide dose, the steady-state concentrations of PB-119, favored by 23 kDa site-specific PEGylation, showed the additional therapeutic advantages of greater non-fasting glucose and HbA1c level reductions and greater insulinotropic activity and better control of diet and body weight in the GK rat model of type 2 diabetes. Site-specific PEGylated exenatide analogs significantly and almost completely diminished systemic anaphylaxis reaction and antigen-specific IgG in guinea pigs. Taken together, site-specific PEGylation is a desirable approach to develop long-acting exenatide based GLP-1 receptor agonists and PB-119 has therapeutic potential as an effective long acting GLP-1 receptor agonist for the treatment of type 2 diabetes mellitus.

Key words: GLP-1 receptor; exenatide; site-specific PEGylation; GK rats; glycaemic control
Nian Gong, PhD Candidate

**King’s Lab**
Shanghai Jiao Tong University School of Pharmacy
No.6 Biomedicine Building (Suite 105)
800 Dongchuan Road, Shanghai 200240, China
Telephone/Fax: 86-21-3420-4766; Mobile: 86-137-9530-4390
Email: niangong0912@yahoo.com
Website: [http://pharm.sjtu.edu.cn/group/king-lab/Default.aspx](http://pharm.sjtu.edu.cn/group/king-lab/Default.aspx)

**EDUCATION**

**PhD, Pharmacology** 2007-Present
Shanghai Jiao Tong University, Shanghai, China

**BSc, Pharmacy** 2003-2007
Xiang Tan University, Xiangtan, Hunan, China

**WORK EXPERIENCE**

**Research Assistant and Assistant Lab Manager** 2008-present
King’s Lab
School of Pharmacy
Shanghai Jiao Tong University
The No. 6 Biomedicine Building (Suite 105)
800 Dongchuan Road
Shanghai 200240, China
Telephone/Fax: 86-21-3420-4766
Website: [http://pharm.sjtu.edu.cn/group/king-lab/Default.aspx](http://pharm.sjtu.edu.cn/group/king-lab/Default.aspx)

**AWARDS AND HONORS**

Hai Ni Pharmaceutical Scholar Award, Shanghai Jiao Tong University, 2013

The 19th Shanghai “Technology Venture Cup”, Shanghai Association of Inventions, 2013

Excellent Poster Award, The 12th Meeting of the Asia Pacific Federation of Pharmacologists, 2013
National Scholarship for Graduate Students, 2012

Predoctoral Fellowship of Shanghai Jiao Tong University, 2012

Annul Graduate Student Presentation Award, Shanghai Jiao Tong University School of Pharmacy, 2012

Scholarship for Excellent Doctoral Student of Ministry of Education, 2011

Predoctoral Fellowship of Shanghai Jiao Tong University, 2011

Predoctoral Fellowship of Shanghai Jiao Tong University School of Pharmacy, 2011

Best Poster Award, The 9th IASP Research Symposium “Understanding Mechanisms of Chronic Pain”, 2011

Best Poster Award, Chinese Pharmacological Society, 2011

An Jie Lun Scholar Award, Shanghai Jiao Tong University, 2011

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Young Scientist Best Paper Award, Shanghai Pharmacological Society, 2010

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