

Dual-acting peptide with prolonged glucagon-like peptide-1 receptor agonist and glucagon receptor antagonist activity for the treatment of type 2 diabetes

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Abstract

Type 2 diabetes is characterized by reduced insulin secretion from the pancreas and overproduction of glucose by the liver. Glucagon-like peptide-1 (GLP-1) promotes glucose-dependent insulin secretion from the pancreas, while glucagon promotes glucose output from the liver. Taking advantage of the homology between GLP-1 and glucagon, a GLP-1/glucagon hybrid peptide, dual-acting peptide for diabetes (DAPD), was identified with combined GLP-1 receptor agonist and glucagon receptor antagonist activity. To overcome its short plasma half-life DAPD was PEGylated, resulting in dramatically prolonged activity *in vivo*. PEGylated DAPD (PEG-DAPD) increases insulin and decreases glucose in a glucose tolerance test, evidence of GLP-1 receptor

agonism. It also reduces blood glucose following a glucagon challenge and elevates fasting glucagon levels in mice, evidence of glucagon receptor antagonism. The PEG-DAPD effects on glucose tolerance are also observed in the presence of the GLP-1 antagonist peptide, exendin(9–39). An antidiabetic effect of PEG-DAPD is observed in *db/db* mice. Furthermore, PEGylation of DAPD eliminates the inhibition of gastrointestinal motility observed with GLP-1 and its analogues. Thus, PEG-DAPD has the potential to be developed as a novel dual-acting peptide to treat type 2 diabetes, with prolonged *in vivo* activity, and without the GI side-effects.

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Introduction

Glucagon-like peptide-1 (GLP-1) is a potent endogenous modulator of insulin secretion that is released by the L-cells in the crypts of the jejunum and ileum in response to a meal (Kieffer & Habener 1999). Its function is to prepare the pancreatic β cells to respond to the increase in calories with the appropriate release of insulin. Importantly, GLP-1 does not provoke hypoglycemia because it does not stimulate insulin secretion unless the plasma glucose concentration is greater than 5 mM (Holz *et al.* 1993). Therapies based on this principle would provide a significant advantage over sulfonylureas (SFUs) and meglitinides that stimulate insulin secretion from pancreatic β -cells through closure of K_{ATP} channels, but whose actions are not glucose-dependent (for review, see Rendell 2004).

No small molecule activators of the GLP-1 receptor have been described. This has led to the development of GLP-1 peptide-based therapeutics. GLP-1 itself has a very short half-life (<5 min) *in vivo* due to degradation by dipeptidyl peptidase-IV (DPP-IV) and clearance by the kidney (Kieffer

et al. 1995). Several inhibitors of DPP-IV, as well as DPP-IV-resistant peptide analogues of GLP-1, are in development for the treatment of type 2 diabetes (Weber 2004 and for review, see Knudsen 2004). One frequently observed side-effect of GLP-1, or its analogues, is nausea and vomiting correlating with the inhibition of gastrointestinal (GI) motility (Buse *et al.* 2004, Elbrond *et al.* 2002). This effect may be mediated, at least partially, by GLP-1 activity in the brain (Imeryuz *et al.* 1997, Wettergren *et al.* 1998).

Glucagon is a 29-amino acid peptide secreted by the α cells of the pancreatic islets in response to reduced blood glucose levels (Unger *et al.* 1978). This hormone travels via the portal blood to the liver where it activates the glucagon receptor. This sets in motion a series of cAMP-mediated events that result in increased hepatic glucose production from both glycogenolysis and gluconeogenesis (Jelinek *et al.* 1993). Glucose production is inappropriately elevated in type 2 diabetes, which is due to insulin resistance in the liver that reduces the ability of insulin to suppress glucose production, and to the continued release of glucagon in the face of hyperglycemia (Baron *et al.* 1987, Shah *et al.* 2000).

In this study, we aimed to target with a single molecule both the defect in insulin secretion and the glucagon-mediated elevation in hepatic glucose production occurring in type 2 diabetes. The feasibility of such an approach is based on the high degree of homology (~50%) between GLP-1 and glucagon (Holst 1996). GLP-1/glucagon hybrid peptides that bind to both receptors have been reported previously (Hjorth *et al.* 1994). However, the agonist or antagonist activity of these hybrid peptides on the GLP-1 or glucagon receptors was not demonstrated. Taking advantage of the high degree of sequence homology between GLP-1 and glucagons, we recently described the identification of a series of GLP-1/glucagon hybrid peptides that had both GLP-1 agonist and glucagon antagonist activity *in vitro* (Pan *et al.* 2006). Here, we report that the optimal hybrid peptide described previously (ANC7K2), which we now refer to as dual-acting peptide for diabetes (DAPD), has both GLP-1 agonist and glucagon antagonist activity *in vivo*.

One approach to prolonging the *in vivo* half-life of a protein is PEGylation, the covalent attachment to a target molecule of long-chained polyethylene glycol (PEG) molecules produced by linking repeating units of ethylene oxide (Harris *et al.* 2001). PEGylation significantly increases the *in vivo* half-life by protecting the protein from protease digestion and by keeping the material out of the kidney filtrate. PEGylation of DAPD with high molecular mass PEG (43 kDa) prolonged the *in vivo* duration of action of the hybrid peptide, and had the added feature of eliminating the GI side-effects.

Materials and Methods

Peptide synthesis and PEGylation

GLP-1, glucagon, FA-GLP-1, and DAPD were supplied by Sigma Genosys or SynPep. The peptides were characterized by HPLC and mass spectrometry and were >90% pure (data not shown). DAPD was site-specifically PEGylated at the C-terminal cysteine with 22, 33, or 43 kDa PEG-maleimide purchased from Nektar Therapeutics (San Carlos, CA, USA) and purified to >95% purity (data not shown).

Animals

Male *BALB/c* mice (20–25 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Male Wistar rats (220–250 g) were purchased from Harlan (Indianapolis, IN, USA). Female *db/db* mice C57BLKS/J *Lepr^{db}/Lepr^{db}* were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 5–6 weeks of age. All animals were maintained on standard laboratory rodent chow available *ad libitum* for at least 5 days before being used in an experiment. All procedures were approved by the Bayer Animal Care and Use Committee and all experiments were performed in accordance with relevant guidelines and regulations.

i.p. Glucose tolerance test (IPGTT)

The IPGTT was performed in male *BALB/c* mice or male Wistar rats as previously described (Pan *et al.* 2006). Briefly, mice or rats were either fasted overnight and then given peptide or vehicle by s.c. injection or they were given peptide or vehicle first and then fasted overnight, depending on the time interval between dosing and when the IPGTT was to be performed. At the appropriate time after dosing, the fasting blood glucose level was measured from tail-tip blood using a Glucometer (Bayer HealthCare) and the animals were given 2 g/kg glucose by i.p. injection. Blood glucose was measured again after 15, 30, and 60 min. The area under the glucose curve (AUC) was calculated using the trapezoidal method, and the effect of the peptide on the AUC was expressed as a percentage of the AUC for the vehicle-treated group. When insulin levels were measured, blood (80–100 µl) was collected by retro-orbital puncture and plasma insulin levels were determined with a mouse insulin ELISA kit (Alpco Diagnostics, Salem, NH, USA).

Gastrointestinal motility in mice and rats

Male *BALB/c* mice or male Wistar rats were either fasted overnight first and then given peptide or vehicle by s.c. injection or they were given peptide or vehicle first and then fasted overnight, depending on the time interval between dosing and the measurement of motility. At the appropriate time after dosing, the mice were given a charcoal meal (10% charcoal, 5% gum Arabic, and 1% carboxymethylcellulose in a volume of 0.8 ml) by oral gavage and then euthanized by cervical dislocation 5 min later. The small intestine was dissected out and the length of the intestine measured as well as the length of the charcoal traveled past the pyloric sphincter. The percentage traveled was calculated by dividing the distance the charcoal traveled by the total length of the small intestine and multiplying by 100.

Gastrointestinal motility in rats following peptide administration by *i.c.v.* injection

Male Wistar rats (275–350 g) were anesthetized with isoflurane gas, and a single 21G stainless steel cannula was aimed at the brain third ventricle using a stereotaxic instrument and the following coordinates: –2.2 mm posterior from the bregma and –7.5 mm ventral to the dura. The cannula was secured to the skull with jeweler's screws and dental cement. One week after surgery, cannula placement was tested by infusion of 1 µl of 10 ng/µl angiotensin II. Animals that drank 5 ml or more of water in an 1-h period were retained for the study. On the day of the gastric motility experiment, 10 µl vehicle (PBS), GLP-1 (7–36) amide peptide, or PEG-DAPD were infused into the third ventricle of overnight fasted rats using an infusion pump (Harvard Apparatus, Holliston, MA, USA). Peptides or vehicle were infused for 2 min and the injection needle was kept in place for an additional minute.

Five minutes post-infusion GI motility was measured as described previously. After the experiment, correct placement of the cannula was verified by injection of Evans Blue followed by brain sectioning.

Glucagon-stimulated glucose production in fed BALB/c mice

Fed male *BALB/c* mice were given a s.c. injection of vehicle (PBS), PEG–GLP-1, or PEG–DAPD and food was withheld. Two hours and forty-five minutes later, the mice were given an i.p. injection of saline or 10 mg/kg somatostatin (SRIF). Fifteen minutes later (zero time), the mice were given another i.p. injection of saline or SRIF plus a s.c. injection of vehicle (0.1% albumin–saline) or 10 µg/kg glucagon. Blood glucose was measured from tail-tip blood using a Glucometer at 0, 15, and 30 min after the glucagon (vehicle) injection. The glucose AUC was calculated as described previously.

Blood glucose measurements in the presence of exendin(9–39) in mice

Male *BALB/c* mice were fasted overnight and then given a s.c. injection of vehicle or various doses of PEG–DAPD or FA–GLP-1, as well as a s.c. injection of PBS or exendin(9–39) (100 µg/kg). The exendin(9–39) or PBS injection was repeated 1 and 2 h later. Three hours after the initial injections, fasting blood glucose was measured from tail-tip blood using a Glucometer. The mice were then given an i.p. injection of 2 g/kg glucose and blood glucose measured 15, 30, 60, and 90 min later.

Blood glucose in db/db mice

Female *db/db* mice C57BLKS/J *Lepr^{db}/Lepr^{db}* (6 weeks old) were fasted overnight and then treated as described under IPGTT. Following the IPGTT, the mice were grouped according to their glucose AUCs and given a s.c. injection of vehicle (PBS), 10 or 30 µg/kg PEG–DAPD. The mice were injected every other day for 2 weeks. Fasting blood glucose was measured 24 h after dosing at 7 and 8 weeks of age.

The blood glucose of 10-week-old female *db/db* mice was determined as described and the mice with equivalent mean values were grouped together. The mice were fasted overnight and their fasting blood glucose determined. The mice were given a s.c. injection of either vehicle (PBS), PEG–DAPD (30 µg/kg), or FA–GLP-1 (30 µg/kg) daily for 2 weeks. Both fed and fasting blood glucose were measured 24 h after dosing at 11 and 12 weeks.

Statistical analysis

Data are expressed as means ± S.E.M. for the number of animals/group indicated. Statistical analyses were performed using GraphPad InStat (GraphPad software, Inc., San Diego, CA, USA). Treatment effects were analyzed by ANOVA with *post hoc* analysis using Tukey–Kramer Multiple Comparisons

test (parametric methods) or Kruskal–Wallis test (non-parametric methods) when necessary. Differences are considered significant at *P* values < 0.05.

Results

Glucose lowering efficacy and gastrointestinal motility in mice

The ability of PEG–DAPD (PEGylated with 43 kDa branched PEG) to increase insulin and decrease glucose *in vivo* was examined by performing an IPGTT in mice 3 h after peptide administration by s.c. injection. PEG–DAPD (30 µg/kg) causes a significant increase in insulin levels, with an 88 ± 16% increase in the insulin AUC (Fig. 1a and b). There is a corresponding decrease in glucose, with a 50 ± 2% decrease in the glucose AUC (Fig. 1c and d).

DAPD, PEG–DAPD, and FA–GLP-1 (fatty acid modified GLP-1, equivalent to Novo Nordisk's NN2211/liraglutide) were then examined for their ability to lower blood glucose following an IPGTT administered either 3 or 17 h after s.c. administration of 100 µg/kg peptide in mice (Fig. 2). When the IPGTT is performed 3 h after peptide administration DAPD, PEG–DAPD, and FA–GLP-1 reduce blood glucose, with decreases in the glucose AUC of 20 ± 6, 40 ± 6, and 29 ± 2% respectively (Fig. 2a and c). Non-modified GLP-1 has a half-life *in vivo* of < 5 min and, as expected, is inactive in an IPGTT performed 3 h following peptide administration (data not shown). PEG–DAPD reduces the glucose AUC to a greater extent than DAPD, indicating prolonged activity. The prolonged activity of PEG–DAPD is further demonstrated by its ability to reduce blood glucose, with a decrease in the glucose AUC of 48 ± 5%, when the IPGTT is performed 17 h following s.c. administration (Fig. 2b and c). This is consistent with the observation that the level of PEG–DAPD measured in plasma samples 3 and 17 h after s.c. administration of 100 µg/kg is 172 and 124 nM respectively. Neither FA–GLP-1 nor non-PEGylated DAPD has any significant effect on the glucose AUC at 17 h due to the short half-life of both peptides *in vivo*.

FA–GLP-1 causes a dose-dependent decrease in the glucose AUC during an IPGTT performed 3 h following peptide administration, with an ED₅₀ of 34 µg/kg and a maximal decrease in the glucose AUC of 34 ± 1% (Fig. 3a). PEG–DAPD also causes a dose-dependent decrease in the glucose AUC, with an ED₅₀ of 7 µg/kg and a maximal decrease in the glucose AUC of 47 ± 1% (*P* < 0.0001 versus FA–GLP-1; Fig. 3b). Therefore, PEG–DAPD is more potent and more efficacious than FA–GLP-1.

The effect of FA–GLP-1 and PEG–DAPD on GI motility was also examined in mice. As expected, FA–GLP-1 decreases GI motility with a potency similar to its effect in the IPGTT. FA–GLP-1 significantly decreases GI motility by 16 ± 1 and 33% ± 4% at doses of 30 and 100 µg/kg respectively (Fig. 3a). Unlike FA–GLP-1, PEG–DAPD (PEGylated with 43 kDa branched PEG) has no significant effect on GI motility in the mouse at doses up to 100 µg/kg (Fig. 3b). However, DAPD

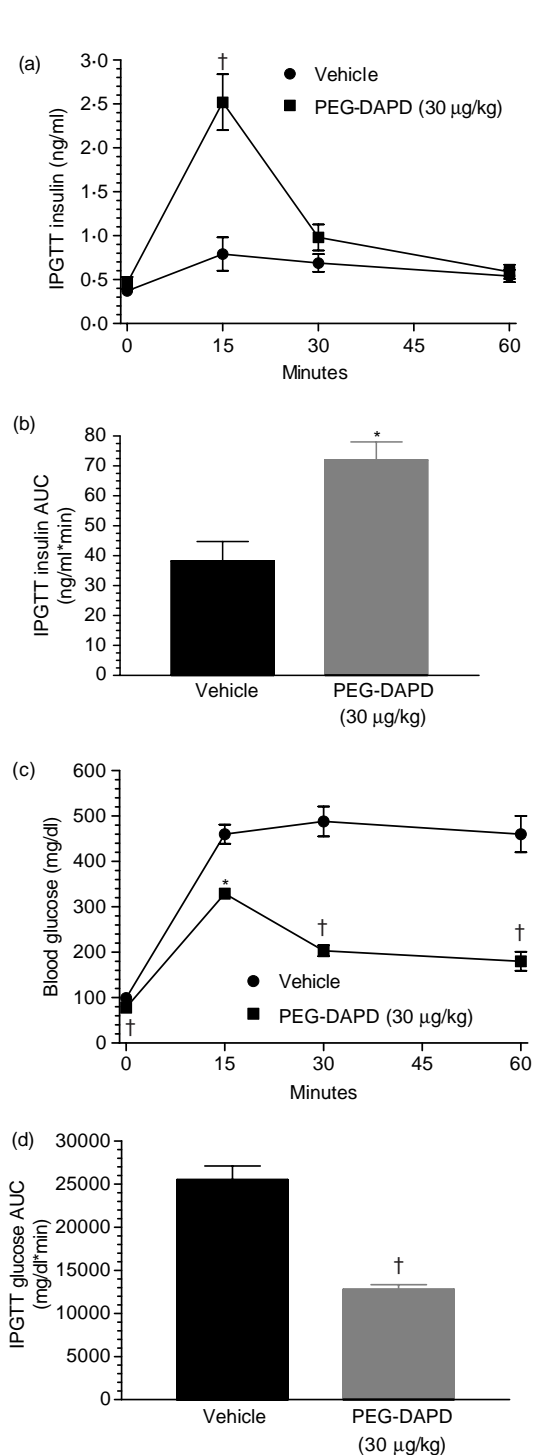


Figure 1 Insulin increasing and glucose lowering activity in an IPGTT in mice. Overnight fasted *BALB/c* mice were injected subcutaneously with vehicle or 30 µg/kg PEG-DAPD and 3 h later an IPGTT was performed. The effect of PEG-DAPD on plasma insulin (a), insulin AUC (b), plasma glucose (c), and glucose AUC (d) was measured. Data are means ± s.e.m. for 8–16 mice/group. * $P < 0.01$ and † $P < 0.001$.

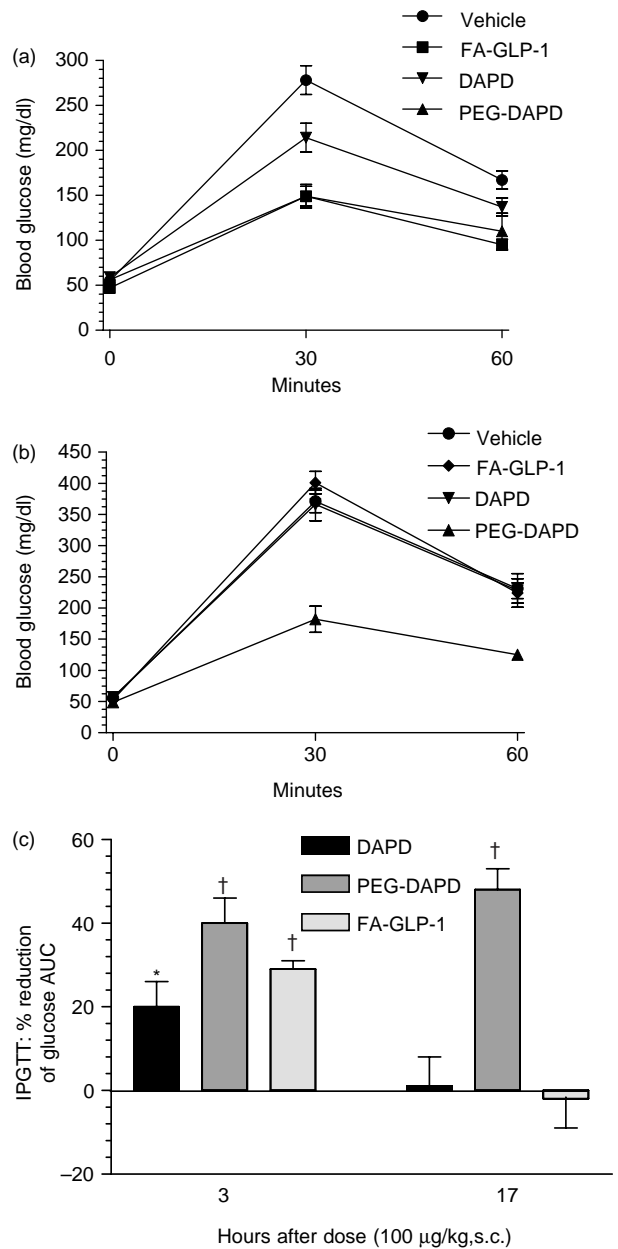


Figure 2 Glucose lowering activity in an IPGTT in mice. Overnight fasted *BALB/c* mice were injected subcutaneously with vehicle or 100 µg/kg FA-GLP-1, PEG-DAPD or DAPD, and either 3 h (a) or 17 h (b) later an IPGTT was performed. The glucose AUC was determined and the percentage reduction due to each treatment was calculated (c). Data are means ± s.e.m. for 8–16 mice/group. * $P < 0.05$ and † $P < 0.001$.

PEGylated with a linear 22 kDa PEG inhibits GI motility with a potency similar to that of FA-GLP-1 (Fig. 3c). DAPD PEGylated with a linear 33 kDa PEG inhibits GI motility to a degree intermediate between that of 22 and 43 kDa PEGylated DAPD. GLP-1 PEGylated with linear 22 kDa PEG inhibits GI motility with a potency similar to that of FA-GLP-1. Similar to

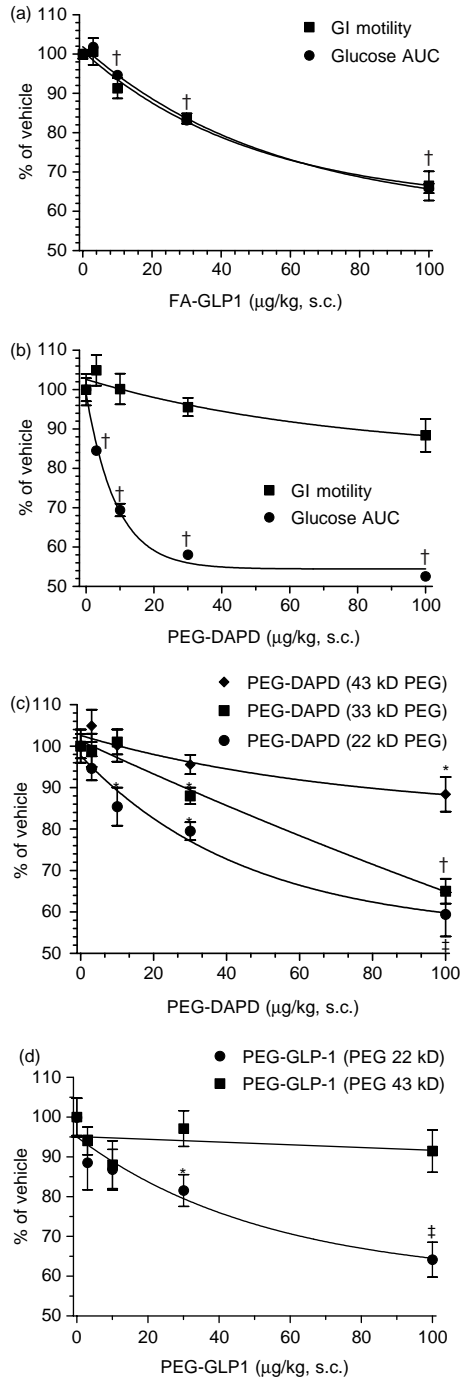


Figure 3 Dose response analysis of glucose lowering activity and gastrointestinal motility effects in mice. Effect of FA-GLP-1 (a) or PEG-DAPD (b) on gastrointestinal motility (■) and glucose AUC (●) in *BALB/c* mice 3 h after peptide administration. Effect of DAPD (c) or GLP-1 (d) PEGylated with PEGs of different molecular mass on gastrointestinal motility in *BALB/c* mice 3 h after peptide administration. Data are means \pm S.E.M. for six mice/group. * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$.

DAPD, GLP-1 PEGylated with branched 43 kDa PEG has no significant effect on GI motility (Fig. 3d).

Glucose lowering efficacy and gastrointestinal motility in rats

The effect of PEG-DAPD on glucose tolerance and GI motility was also examined in rats (Fig. 4). PEG-DAPD causes a dose-dependent decrease in blood glucose in an IPGTT performed 17 h after s.c. administration of the peptide. The decrease in the glucose AUC is 11 ± 3 and $19 \pm 6\%$ at doses of 1 and 3 $\mu\text{g/kg}$ respectively. A maximal decrease in the glucose AUC is observed at a dose of 3 $\mu\text{g/kg}$ as similar decreases are observed at doses up to 100 $\mu\text{g/kg}$ (Fig. 4a). Therefore, PEG-DAPD is more potent, but less efficacious, in an IPGTT in rats when compared with mice, as is FA-GLP-1 (data not shown). PEG-DAPD has no significant effect on GI motility in rats at doses up to 100 $\mu\text{g/kg}$ (Fig. 4a), whereas FA-GLP-1 decreases GI motility in rats with efficacy and potency similar to its effect in an IPGTT (data not shown).

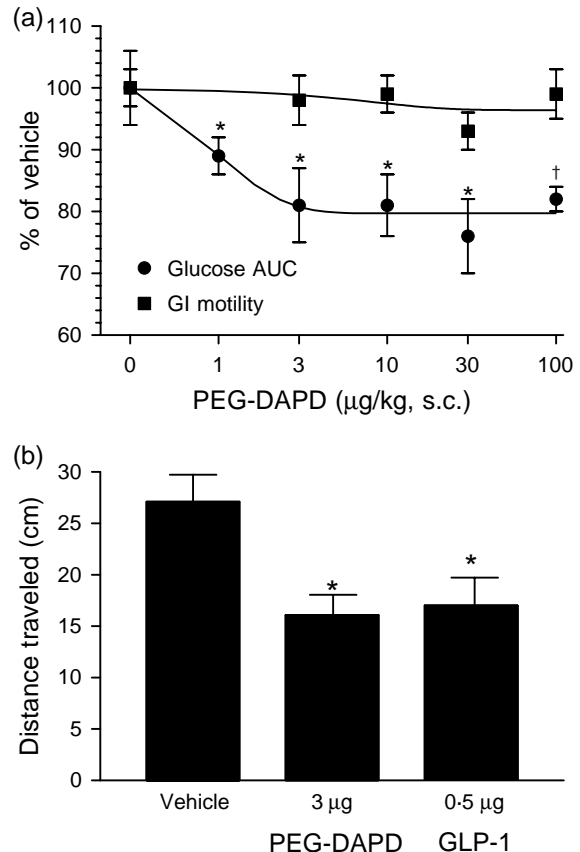


Figure 4 Glucose lowering activity and gastrointestinal motility effects in rats. (a) Effect of PEG-DAPD on glucose AUC in an IPGTT in fasted Wistar rats (●) and on gastrointestinal motility (■) 17 h after peptide administration. The data are means \pm S.E.M. for eight and six rats/group respectively. (b) Effect of CNS administered PEG-DAPD and GLP-1 on gastrointestinal motility. The data are means \pm S.E.M. for ten rats/group. * $P < 0.05$ and † $P < 0.01$.

An IPGTT was performed at 17, 41, 65, and 89 h following s.c. administration of a single 30 µg/kg dose of PEG-DAPD in rats. PEG-DAPD administration up to 65 h prior to the IPGTT results in a significant decrease in the glucose AUC (Table 1). There is also a trend towards a decrease at 89 h after dosing. These results demonstrate that PEG-DAPD has dramatically prolonged activity when compared with non-modified GLP-1 because of the improved stability and reduced clearance of the PEGylated peptide.

Attachment of a 43 kDa PEG (branched) to DAPD may restrict its ability to cross the blood–brain barrier and thereby account for its inability to affect GI motility. In support of this hypothesis, central administration of 3 µg/kg PEG-DAPD by intracerebroventricular (i.c.v.) injection results in a significant reduction in GI motility, similar to that observed with 0.5 µg/kg GLP-1 (Fig. 4b).

Inhibition of glucagon action in mice

PEG-DAPD could mediate a reduction in the glucagon-induced increase in blood glucose by either direct glucagon receptor antagonism, indirectly through enhanced insulin secretion from the pancreas, or a combination of both. Glucagon antagonist activity of PEG-DAPD *in vivo* was examined first by performing a glucagon challenge in mice and comparing the activity of PEG-DAPD with that of PEG-GLP-1 (GLP-1 PEGylated with 43 kDa PEG) on blood glucose levels. PEG-GLP-1 acts specifically as a GLP-1 receptor agonist and does not have glucagon antagonist activity. PEG-DAPD (10 µg/kg) significantly suppresses the glucagon-induced increase in blood glucose AUC, whereas PEG-GLP-1 at the same dose has no effect (Fig. 5a). This indicates that PEG-DAPD has glucagon antagonist activity *in vivo* and it is likely to be, at least in part, directly on the glucagon receptor. In order to explore further the direct glucagon receptor antagonist activity of PEG-DAPD, a glucagon challenge was performed in the presence of 10 mg/kg somatostatin (SRIF), which inhibits pancreatic insulin secretion. PEG-DAPD significantly reduces the glucagon-mediated increase in the glucose AUC (Fig. 5b), whereas PEG-GLP-1 has no significant effect (Fig. 5c). We conclude that the PEG-DAPD mediated reduction in glucose levels in a glucagon challenge reflects direct glucagon receptor antagonist activity *in vivo*.

Table 1 Glucose lowering efficacy of PEG-DAPD in rats when administered subcutaneously (30 µg/kg) at increasing lengths of time prior to an IPGTT. The data are the means ± S.E.M. of 9–16 rats/group

	Hours after dosing PEG-DAPD			
	17 h	41 h	65 h	89 h
Efficacy	24 ± 5	17 ± 2	18 ± 2	11 ± 4
P value	0.001	0.0005	0.0017	0.0905

Efficacy, % reduction of glucose AUC.

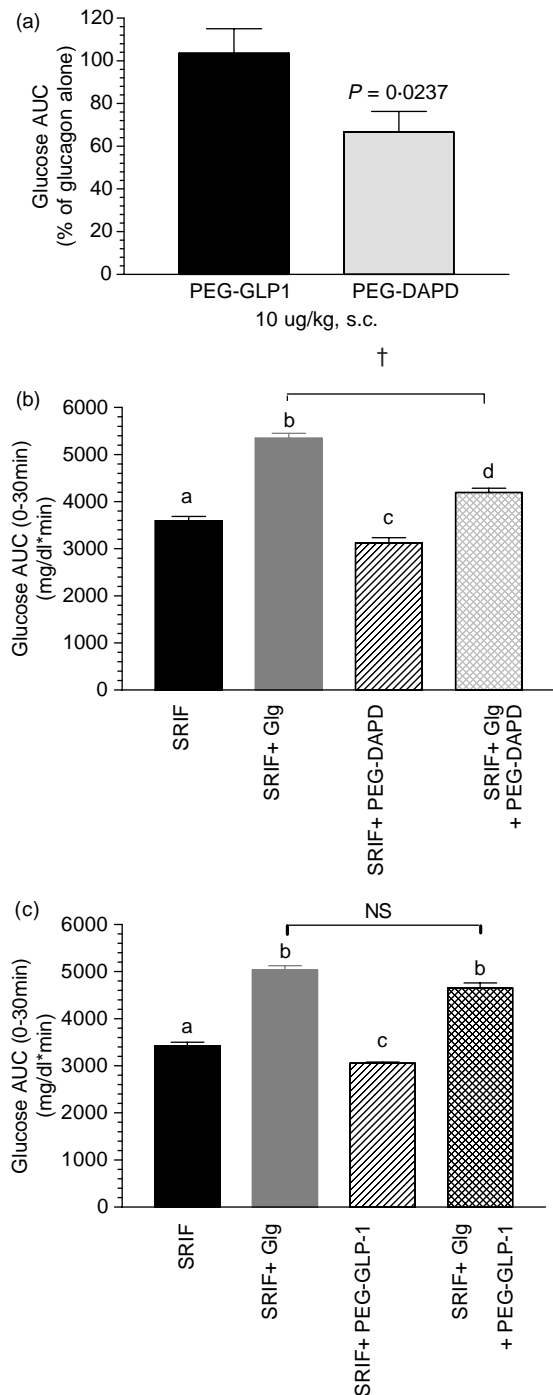


Figure 5 Effect of PEG-DAPD and PEG-GLP-1 on glucagon induced increase in blood glucose in mice. *BALB/c* mice were treated 3 h prior to a glucagon (Glg) challenge with either vehicle, PEG-DAPD (10 µg/kg) or PEG-GLP-1 (10 µg/kg) in the absence (a) or presence (b and c) of somatostatin (SRIF). Somatostatin was administered 15 min prior to, and again at the time of, the glucagon challenge. Data are means ± S.E.M. of 12 mice/group. † $P < 0.001$, NS, not significant. Bars with different letters are significantly different from each other ($P < 0.05$ or less).

Plasma glucagon levels and effect of exendin(9–39) on blood glucose in mice

In order to further assess the glucagon receptor antagonist activity of PEG-DAPD *in vivo* plasma glucagon levels were measured in mice 3 h following PEG-DAPD administration. PEG-DAPD significantly increases plasma glucagon levels at all doses tested (Fig. 6a). This is consistent with reports that knockdown of glucagon receptor expression leads to an increase in plasma glucagon levels (Parker *et al.* 2002, Gelling *et al.* 2003, Liang *et al.* 2004, Sloop *et al.* 2004). On the other hand, GLP-1 agonism decreases glucagon secretion (Nauck *et al.* 1993). Unlike PEG-DAPD, FA-GLP-1 does not significantly affect glucagon levels, although there is a trend towards a decrease at the 100 µg/kg dose (Fig. 6a).

Exendin(9–39) acts as an antagonist of the GLP-1 receptor (Kolligs *et al.* 1995). The effect of exendin(9–39) on the ability of PEG-DAPD and FA-GLP-1 to lower blood glucose during an IPGTT also was examined (Fig. 6b). The PEG-DAPD mediated decrease in the glucose AUC at 10 and 30 µg/kg is only partially reversed by exendin(9–39). This supports a role for glucagon antagonism, as well as GLP-1 agonism, in the reduction of the glucose AUC. PEG-DAPD at 100 µg/kg overcomes the inhibitory effect of exendin(9–39). Exendin(9–39) almost completely inhibited the effect of FA-GLP-1, except at the 200 µg/kg dose. As in the absence of exendin(9–39) (Fig. 3), the maximum decrease in the glucose AUC obtained with PEG-DAPD ($47 \pm 1\%$) is greater than that obtained with FA-GLP-1 ($34 \pm 1\%$; $P < 0.0001$). The results support the conclusion that PEG-DAPD affects glucose homeostasis beyond that which occurs through GLP-1 receptor activation.

Glucose lowering efficacy in db/db mice

Obese diabetic (*db/db*) mice begin to display fasting hyperglycemia at about 8 weeks of age. In order to determine whether PEG-DAPD prevents or delays the onset of hyperglycemia, 6-week-old prediabetic female *db/db* mice were dosed subcutaneously with 10 or 30 µg/kg PEG-DAPD for 2 weeks. Fasting blood glucose levels in the vehicle-treated mice began to rise between 7 and 8 weeks of age, and PEG-DAPD prevented the increase (Fig. 7a). Therefore, PEG-DAPD delays the onset of hyperglycemia in young *db/db* mice. PEG-DAPD also produces a significant decrease in fasting blood glucose in 10–12-week-old diabetic *db/db* mice, while FA-GLP-1 treatment does not, although there was a trend towards a decrease (Fig. 7b). Neither PEG-DAPD nor FA-GLP-1 has any effect on fed blood glucose levels (data not shown).

Discussion

We have identified a novel PEGylated GLP-1 agonist/glucagon antagonist peptide (PEG-DAPD) with a number of attractive features. First, it has both GLP-1 agonist and

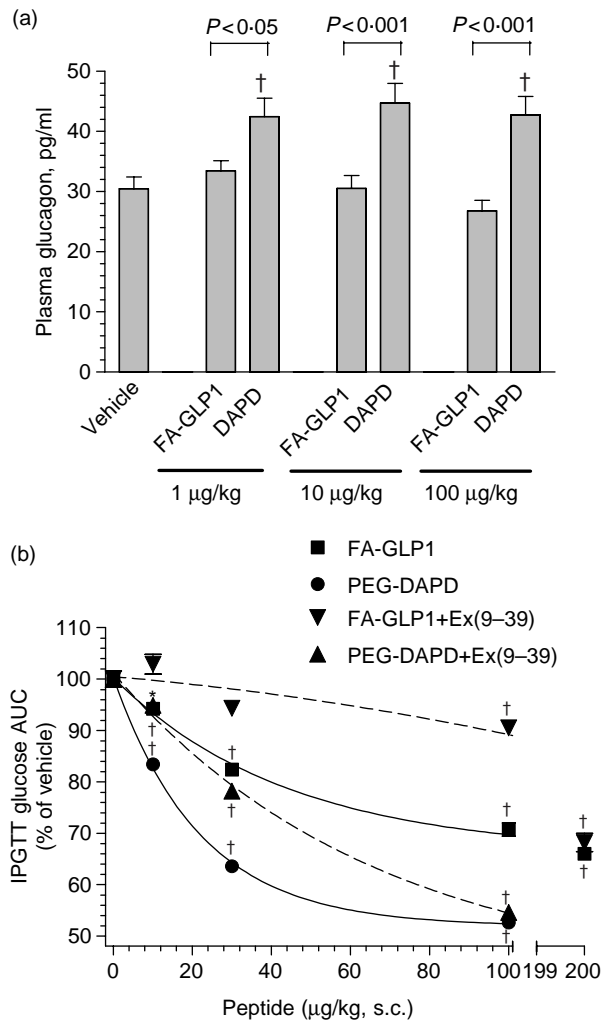


Figure 6 Effect of PEG-DAPD and FA-GLP-1 on plasma glucagon and blood glucose in the presence of exendin(9–39) in mice. Overnight fasted *BALB/c* mice were treated with either vehicle, PEG-DAPD or FA-GLP-1 3 h prior to measurement of plasma glucagon levels (a), or blood glucose in an IPGTT in the presence or absence of exendin(9–39) (Ex (9–39)) (b). Data are means \pm s.e.m. of 8–16 mice/group. * $P < 0.05$ and $^{\dagger}P < 0.01$. In (a) the P values indicated by † refers to PEG-DAPD versus vehicle and the P values shown in the figure refer to PEG-DAPD versus FA-GLP-1 (same dose).

glucagon antagonist activity to lower blood glucose levels. Secondly, through PEGylation and elimination of the DPP-IV cleavage site, the peptide has prolonged activity *in vivo*. Thirdly, PEGylation with high molecular mass (43 kDa) branched PEG eliminates the inhibitory effect on GI motility normally associated with GLP-1, and which is likely to be related to the nausea and vomiting in humans reported with existing GLP-1 agonists (for review, see Drucker 2006).

The properties of GLP-1 make it attractive as a potential therapy for the treatment of type 2 diabetes. Infusion of GLP-1 is effective in lowering blood glucose, even in diabetic

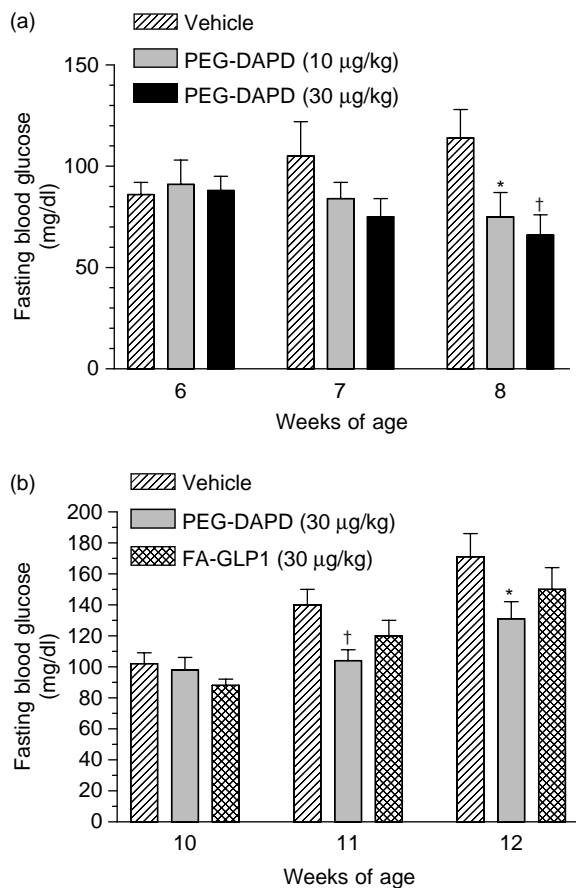


Figure 7 Effect of PEG-DAPD blood glucose in *db/db* mice. (a) Prediabetic *db/db* mice were dosed every other day and fasting blood glucose was measured weekly. (b) Diabetic *db/db* mice were dosed daily and fasting blood glucose was measured weekly. The fasting glucose values are means \pm S.E.M. for 6–8 mice per group. * $P < 0.05$ and † $P < 0.01$ versus vehicle.

patients who have failed on SFUs (Rachman *et al.* 1997, Nauck *et al.* 1998, Toft-Nielsen *et al.* 1999, Zander *et al.* 2002). In addition to promoting glucose-dependent insulin secretion, with little risk of hypoglycemia, GLP-1 also has the potential to preserve β -cell function and delay or even prevent disease progression (Stoffers *et al.* 2000, Egan *et al.* 2003). A GLP-1 analogue is already available for the treatment of type 2 diabetes (Exenatide/Byetta), and others are presently in various stages of development (for review, see Knudsen 2004). These analogues are aimed primarily at overcoming the very short half-life of GLP-1 *in vivo*. Exenatide (Byetta) is administered twice daily as a s.c. injection and demonstrates glucose lowering activity in type 2 diabetics (Fineman *et al.* 2003, Kolterman *et al.* 2003). Transient nausea has also been reported (Blonde *et al.* 2006).

In addition to the GLP-1 agonist activity of PEG-DAPD, its ability to suppress glucagon action is another important property of this peptide. Several lines of evidence support the glucagon antagonism of PEG-DAPD *in vivo*. It reduces blood

glucose following a glucagon challenge, and elevates fasting glucagon levels. The effects of PEG-DAPD on glucose tolerance are also observed in the presence of the GLP-1 receptor antagonist, exendin(9–39). The effect of PEG-DAPD to reduce blood glucose following a glucagon challenge is also observed in the presence of SRIF, which blocks insulin secretion. It has been demonstrated that the reduction in expression of the glucagon receptor, using an antisense oligonucleotide approach, leads to a reduction in blood glucose levels and improved glucose tolerance (Liang *et al.* 2004, Sloop *et al.* 2004). Small molecule glucagon receptor antagonists have been pursued as an approach to treat type 2 diabetes. The most advanced compound reported to date, BAY 27-9955, lowers plasma glucose levels during a hyperglucagonemic period in humans (Petersen & Sullivan 2001). Peptide antagonists of the glucagon receptor have also been described but they have short half-lives *in vivo* (for review, see Sloop & Michael 2004).

Plasma glucagon levels are elevated in *db/db* mice and glucagon plays an important role in their age-associated development of hyperglycemia (Kodama *et al.* 1994). The ability of PEG-DAPD to delay the onset of hyperglycemia in pre-diabetic *db/db* mice and to lower the fasting blood glucose levels in diabetic *db/db* mice supports the dual-acting role of PEG-DAPD to increase insulin secretion and inhibit glucagon action. Other GLP-1 analogues have been reported to lower blood glucose in fed *db/db* mice (Rolin *et al.* 2002, Kim *et al.* 2003). We believe that the effects observed on fed glucose levels in *db/db* mice may be a consequence of the effects of these GLP-1 analogues on food intake at the high doses administered. Alternatively, a recent report suggests that during hyperglycemia central GLP-1 activity plays a role in control of blood glucose (Knauf *et al.* 2005). The present data indicate that PEG-DAPD cannot cross the blood–brain barrier, thus PEG-DAPD may have GLP-1 signaling activity distinct from other GLP-1 analogues to control blood glucose.

PEGylation is an established approach for improving the pharmacokinetic and pharmacodynamic properties of protein pharmaceuticals. The PEG polymer protects the attached protein from enzyme degradation and rapid renal clearance, thereby prolonging the *in vivo* half-life. PEG also reduces interactions with cell surface proteins, thereby limiting adverse immunological effects. As a consequence, a number of PEGylated protein drugs have been approved with properties that lead to significant clinical benefits, such as sustained blood levels, reduced adverse reactions, and improved patient convenience (for review, see Harris & Chess 2003). While proteins that have been PEGylated with high molecular weight PEG and that retain significant *in vivo* activity have been reported, we believe PEG-DAPD represents the first reported PEGylation of a small peptide that retains prolonged *in vivo* activity.

PEG-DAPD was efficacious for as long as 65 h after a single s.c. dose in rats. Therefore, PEG-DAPD could have the potential for infrequent dosing in humans. Given the high prevalence of gastric motility disorders in patients with

diabetes, further inhibition of gastric emptying in these patients is likely to exacerbate symptoms of nausea and vomiting (Horowitz *et al.* 2002). Furthermore, delaying gastric emptying may also alter the pharmacokinetic properties of other medications. PEG-DAPD did not inhibit GI motility when given subcutaneously, but did when given centrally to rats. This supports the hypothesis that PEG-DAPD does not cross the blood–brain barrier, and thereby prevents the peptide from interacting with brain GLP-1 receptors, which mediate, at least in part, the effects on GI motility. The absence of effects of PEG-DAPD on GI motility, if confirmed in humans, has the potential to be of benefit in the clinical setting.

It is reported that the GLP-1 mediated delay in gastric emptying, thereby slowing nutrient entry into the circulation, is an important component of its overall effect on post-prandial glucose homeostasis (Nauck *et al.* 1997, Meier *et al.* 2005). As PEG-DAPD does not inhibit GI motility, it does not have the capability to lower glucose by this mechanism. However, the efficacy of PEG-DAPD in the animal models tested indicates that the glucose lowering activity of PEG-DAPD is at least equivalent, if not superior, to that of FA-GLP-1. Therefore, the absence of glucose lowering by the GI-inhibitory mechanism with PEG-DAPD is most likely compensated for by the glucose lowering arising through its glucagon antagonist action.

With the increasing prevalence of type 2 diabetes and the limitations of the currently available therapies, there is a clear need for new and effective approaches to treat this disease. PEG-DAPD, by acting on two key pathways central to control of glucose homeostasis, GLP-1 and glucagon signaling, potentially represents such an approach. Furthermore, PEGylation, in addition to dramatically prolonging the *in vivo* activity of PEG-DAPD, most likely restricts its access to the central nervous system, thereby eliminating centrally mediated GI effects. Overall, PEG-DAPD has several attractive features which justify pursuing it as a potential treatment of type 2 diabetes.

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