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A once-monthly GLP-1 receptor agonist for treatment of diabetic cats

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ABSTRACT

There is growing evidence that peptidic glucagon-like peptide-1 receptor agonists (GLP-1RA), such as exenatide, may provide useful therapeutic options for treatment of feline diabetes. However, because such drugs are administered subcutaneously, it is desirable that they be long-acting and not require frequent injections. We have developed a chemically controlled delivery system to support half-life extension of peptidic therapeutics. Here, the peptide is covalently attached to hydrogel microspheres by a self-cleaving β-eliminative linker; after subcutaneous injection of the microspheres, the peptide is slowly released from the depot to the systemic circulation. Using this technology, we developed a delivery system that supports once-monthly administration of a stable exenatide analog, [Gln^{28}]exenatide, in rodents (Schneider, et al, ACS Chem Biol 12, 2107 to 2116, 2017). The purposes of the present study were a) to demonstrate pharmacokinetic and pharmacodynamic similarities of the deamidation-sensitive GLP-1RA exenatide and the closely related, more stable [Gln^{28}]exenatide and b) to develop a long-acting GLP-1RA in cats. The results show that exenatide and [Gln^{28}]exenatide injected intravenously or subcutaneously at 10 μg/kg have nearly identical pharmacokinetics in the cat—both having elimination half-lives of ~40 min—but subcutaneously administered [Gln^{28}]exenatide has superior bioavailability—93% for [Gln^{28}]exenatide vs 52% for exenatide. The results also show that exenatide and [Gln^{28}]exenatide have similar insulinotropic activities in the cat during a high-dose intravenous glucose tolerance test; they increased the area under the curve (AUC) for insulin to a similar extent but had no effect on glucose AUC. Finally, subcutaneous injection of a microsphere-[Gln^{28}]exenatide conjugate containing an appropriate self-cleaving linker in the cat provides plasma [Gln^{28}]exenatide with a half-life of about 40 d vs 40 min with the injected free peptide. Hence, the large body of information available for exenatide can be used to facilitate clinical development of [Gln^{28}]exenatide as a treatment for feline diabetes, and the microsphere-[Gln^{28}]exenatide conjugate is quite suitable for once-monthly subcutaneous administration of the peptide in the cat.

1. Introduction

It has been estimated that there are well over 150,000,000 domestic cats in the United States and Europe [1,2]. Diabetes mellitus (DM) is a common endocrine disease of cats, which is believed to closely resemble human type 2 diabetes (T2D) in both etiology and clinical presentation. In a study of almost 200,000 cats in England over September 1, 2009, to August 31, 2014, it was shown that the prevalence of diabetes in cats was 0.58% or about 1 in 200 [3], which translates to over 750,000 diabetic cats in the United States and Europe.

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Currently, the most common treatments for DM in cats are dietary changes and insulin injections, with the common dosing regimen of insulin being subcutaneous (SC) twice daily. Although there are 2 branded insulins (Prozinc and Vetsulin) approved and marketed for cats, many owners may treat their cats with human insulin [4], and because sales of insulin for vet use are unreported, it is difficult to determine the number of cats that are actually treated with medication vs dietary changes alone or are left untreated to suffer the disease.

The glucagon-like peptide-1 receptor agonists (GLP-1RAs) are relatively new therapeutics used to treat T2D in humans. Glucagon-like peptide-1 receptor agonists have a glucose-dependent insulinotropic effect [5–7], that is, in the presence of high glucose, they stimulate the pancreatic β-cell to secrete and synthesize insulin, and they likely help preserve β-cell mass [8]. When effective, GLP-1RAs control blood glucose with minimal side effects, delay gastric emptying, and reduce eating and BW [5,7,9]. Glucagon-like peptide-1 receptor agonists have more recently been shown to be cardioprotective in long-term studies in diabetic patients [10,11].

Several studies have shown that GLP-1RAs—such as the 39 amino acid peptide exenatide —are quite safe and show insulinotropic effects in normal cats; it has been suggested that these agonists might serve a role in treating cats with T2D [12–15]. In particular, they cause robust insulin secretion from pancreatic β-cells when animals are provided a bolus of glucose in various glucose tolerance tests [12,13,16]. There have been fewer studies that investigated the effects of GLP-1RAs in cats with diagnosed DM [14,17] and whether they show long-term effectiveness as single therapy in treating the disease in felines remains an open question.

A potential major advantage of using GLP-1RAs instead of twice-daily SC insulin in treatment of cat diabetes is that several long-acting agonists are available that require much fewer injections of the drug. Currently, there are 3 marketed once-weekly (QW) GLP-1RAs for treatment of human diabetes [11,18], but whether the same pharmacokinetics and pharmacodynamics would translate to cats is unknown. If so, 14 injections of insulin per week could be replaced by a single injection of a GLP-1RA.

We have developed a general approach for half-life extension of therapeutics in which a drug is covalently tethered to a long-lived carrier by a linker that slowly cleaves by β-elimination to release the native drug (Scheme 1) [19,20]. The cleavage rate of the linker is controlled by the nature of an electron-withdrawing "modulator" (Mod) attached to a carbon containing an acidic C–H bond. These linkers are not affected by enzymes and are stable for years when stored at low pH and temperature [20].

One carrier we use is a large-pore tetra-PEG hydrogel polymer [19,21]. These hydrogels—fabricated as uniform ~40 μm microspheres—[22] are injected subcutaneously through a small-bore needle where they serve as a depot to slowly release the drug to the systemic circulation. We also incorporate slower cleaving eliminative linkers in crosslinks of these polymers, so gel degradation occurs after drug release.

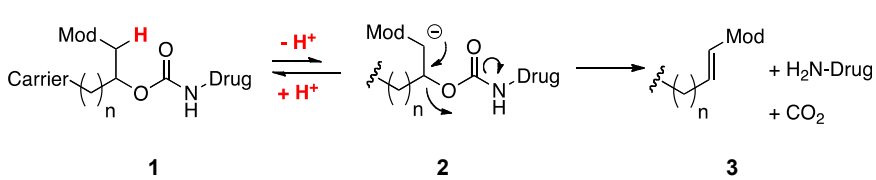
We previously reported a releasable tetra-PEG hydrogel-exenatide conjugate that after SC administration provides systemic exenatide with a $t_{1/2,\beta}$ of ~7 d in the rat and supported QW administration [22]. This conjugate is comparable to current QW GLP-1 agonists, but the several weekly agonists already on the market reduce impetus for its clinical development. Thus, we prepared and studied an analogous hydrogel-exenatide conjugate that had a linker expected to release the peptide with an in vivo half-life of 1 mo [23]. However, we unexpectedly found that exenatide undergoes deamidation at Asn^{28} with an in vitro and in vivo half-life of approximately 2 wk. We thus prepared [Gln^{28}]exenatide, which was stable for long periods. [Gln^{28}] exenatide and exenatide showed indistinguishable GLP-1R agonist activities as well as pharmacokinetic and pharmacodynamic effects in rodents; when attached to microspheres by an appropriate β-eliminative linker, a delivery system for once-monthly administration was achieved that was equieffective to a continuous infusion of exenatide in a rat model of T2D [23].

In the present work, we describe studies in the cat that a) demonstrate that the pharmacokinetics of [Gln^{28}]exenatide are essentially identical to that of exenatide, b) demonstrate that the insulinotropic effect of the analog is similar to exenatide, and c) show that the microsphere-peptide conjugate serves as an effective drug reservoir that provides [Gln^{28}]exenatide with a half-life suitable for once-monthly administration. If this once-monthly GLP-1RA were clinically effective, some 56 BID insulin injections per month in the cat could be replaced by a single injection of the long-acting drug.

2. Materials and methods

2.1. General

UV analyses were performed using a Hewlett-Packard 8453 UV-Vis spectrophotometer or a Thermo Scientific NanoDrop 2000 spectrophotometer. Concentrations of peptide solutions were determined using ε_{280} = 5,500 M^{-1} cm^{-1} for the single tryptophan present. ELISA assays were read on a Molecular Devices SpectraMax i3 plate



Scheme 1. β-eliminative drug release from linker.

reader. O-(7-Azido-1-cyano)-O′-succinimidyl carbonate was prepared as reported in the study by Santi et al [20].

2.2. Synthesis of azido-linker-[Gln^{28}]exenatide

Peptide synthesis and linker attachment were performed as described by Schneider et al [22,23].

2.3. Microsphere-[Gln^{28}]exenatide conjugate

The 40-μm microsphere-[Gln^{28}]exenatide conjugate was made and handled exactly as described by Schneider et al [22], except 5-hydroxy cyclooctyne (5HCO)-HSC [24] was used to activate the microspheres for strain-promoted alkyne-azide cycloadditions instead of monofluoro-substituted cyclooctyne pentafluorophenyl ester.

2.3.1. O-Cyclooct-4-yn-1-yl-O′-succinimidyl carbonate (5-HCO-HSC)

A 250-mL, round-bottomed flask was charged with 5-hydroxy cyclooctyne [24] (3.3 g, 26.6 mmol, 1.0 equiv, 0.2 M final concentration), CH_2Cl_2 (122 mL), N,N'-Disuccinimidyl carbonate (13.6 g, 53.2 mmol, 2 equiv, 0.4 M final concentration), Et_3N (8.11 mL, 58.5 mmol, 2.2 equiv, 0.4 M final concentration), and 4-N,N-Dimethylaminopyridine (650 mg, 5.32 mmol, 0.2 equiv, 0.04 M final concentration). The reaction mixture was stirred at ambient temperature for 24 h. The reaction mixture was diluted with CH_2Cl_2 (300 mL) and washed with saturated $NaHCO_3$, H_2O, 5% aqueous $KHSO_4$, H_2O, brine (100 mL each). The organic phases were concentrated and filtered through a cotton plug onto a silica gel column (120 g silica gel cartridge). Gradient elution (10%, 20%, 30%, 40%, 50% [200 mL each] acetone/hexanes) afforded 6.73 g (88% purity by weight; by ^{1}H NMR = 5.92 g, 22.3 mmol, 84% calculated yield) of desired HSC as a yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 4.79 (dd, J = 9.4, 5.3 Hz, 1 H), 2.81 (s, 4 H), 2.28 to 2.56 (m, 2 H), 2.04 to 2.28 (m, 6 H), 1.82 to 2.03 (m, 2 H). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.8, 150.9, 94.1, 92.8, 87.8, 39.6, 37.8, 30.8, 29.8, 25.4, 19.9, and 17.5.

2.3.2. Microsphere-[Gln^{28}]exenatide conjugate

To a suspension of 16.4 g of a slurry of the 5HCO-derivatized microspheres (46.8 μmol 5HCO) in 100 mM citrate in 1:1 DMSO:H_2O at pH 5.0 in a 250-mL conical centrifuge tubes was added a solution of 324 mg (72 μmol) of N$^{\alpha}$-(7-Azido-1-cyano-2-heptyloxycarbonyl)-[Gln^{28}]exenatide in 16 mL of 100 mM citrate in 1:1 DMSO:H_2O at pH 5.0. The mixture was slowly rotated until the OD_{280} of an aliquot was constant at ~24 h. The microsphere slurry was pelleted, and the reaction supernatant was decanted off. The microspheres were washed with 5 × 200 mL of 100 mM citrate in 1:1 DMSO:H_2O at pH 5.0 followed by 5 × 200 mL of isotonic acetate (10 mM sodium acetate, 143 mM NaCl), pH 5.0, and 0.05% Tween 20. The total loading of the microsphere was 2.3 μmol [Gln^{28}]exenatide gm^{-1} of slurry as determined by measurements of the total peptide released in 50 mM NaOH by A_{280} (ε_{280} = 5,500 M^{-1} cm^{-1}) and by PEG analysis of the NaOH-solubilized microspheres [25].

2.4. Pharmacokinetics of exenatide and [Gln^{28}]exenatide

All pharmacokinetic studies were performed by Amatsigroup, Inc (Terre Haute, IN).

2.4.1. Pharmacokinetics of exenatide and [Gln^{28}]exenatide

Solutions of exenatide and [Gln^{28}]exenatide at 500 μg/mL, determined by A_{280} (ε_{280} = 5,500 M^{-1} cm^{-1}), were prepared in sterile 10 mM NaOAc, pH 4.5, and filtered through a sterile, centrifugal spin filter (0.2 μm). Male domestic short-haired (DSH) cats aged 12 to 48 mo and ranging between 3.7 and 6.8 kg (mean = 5.2 ± 0.9 kg) with cephalic cannulas were dosed at 10 μg/kg exenatide or [Gln^{28}]exenatide intravenously through the cephalic cannula (n = 6 cats for each peptide), and the catheter was flushed with 1 mL of saline. Blood samples (~1.5 mL) were drawn before dosing and at 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, and 180 min after dosing from the jugular vein. Plasma was collected using 3 mL EDTA (purple top) Vacutainer tubes containing 30 μL of 50X protease inhibitor cocktail (Sigma #P2714) and frozen at −80°C until analysis. Plasma exenatide and [Gln^{28}]exenatide were analyzed by LC/MS/MS as described below. The C vs t plots were obtained using GraphPad Prism 8 for Mac by nonlinear regression using the exponential 2-phase decay model found within the program with weighting by 1/SD^2.

2.4.2. Pharmacokinetics of the hydrogel-microsphere [Gln^{28}] exenatide conjugate

A total of 12 male DSH cats aged 12 to 48 mo and ranging between 3.7 and 6.8 kg (mean = 5.3 ± 0.9 kg) of BW were used in the study. Syringes were filled with microspheres under aseptic conditions (3 mL Luer-Lok tip, BD # 309657, or 1 mL Soft-Ject Luer-Lok tip, Henke Sass Wolf #2021-05) with [Gln^{28}]exenatide-microsphere slurry in isotonic acetate (10 mM sodium acetate, 143 mM NaCl), pH 5.0, and 0.05% Tween 20. The microspheres contained 2.3 μmol [Gln^{28}] exenatide gm^{-1} of slurry. The contents in each syringe were administered through a 21-g needle subcutaneously in the periscapular area. Groups of 6 cats were dosed with either 2 g of slurry containing 19.3 mg [Gln^{28}]exenatide (4.6 μmol) or 0.5 g of slurry containing 4.8 mg [Gln^{28}]exenatide (1.2 μmol). Over the study period, the cats were monitored for signs of irritation on the injection site, inappetence, diarrhea, and emesis. For pharmacokinetics, blood samples (~1.5 mL) were drawn before dosing and at 4, 8, 24, 48, 72, and 12 h and at 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 d after dosing from the jugular, cephalic, or femoral vein with the sites alternating between collections. Approximately 1.5 mL of plasma was collected using 3 mL EDTA (purple top) Vacutainer tubes containing 30 μL of 50X protease inhibitor cocktail (Sigma #P2714). The plasma samples were prepared and frozen at −80°C until analysis. Plasma [Gln^{28}] exenatide was analyzed by LC/MS/MS as described below. For each cat, a normalized concentration, C_N, was calculated as $C_N = C_m(D_{ave}/D_{inj})$ where C_m is the measured concentration, D_{ave} is the average dose given to all cats, and D_{inj} is the dose injected in the subject cat. The C vs t plots were obtained using GraphPad Prism 8 for Mac by non-linear regression using a user-defined bi-exponential equation [23] and weighting by 1/SD^2.

2.5. Pharmacodynamics of exenatide vs [Gln^{28}]exenatide

2.5.1. Intravenous glucose tolerance test after exenatide and [Gln^{28}]exenatide administration

The intravenous glucose tolerance test (IVGTT) study was run at Amatsigroup, Inc (Terre Haute, IN). A total of 30 male DSH cats aged 12 to 48 mo and ranging between 4.0 and 6.8 kg (mean = 5.5 ± 0.8 kg) BW were used in the study. On the day before the experiment, the cats were stratified according to BW into 5 groups of n = 6 and fasted for 12 h before glucose bolus. On the day of the experiment, jugular and cephalic catheters were placed on each cat 180 min before glucose administration. Each cat was placed in a gas chamber, and anesthesia was induced with isoflurane gas. Once anesthetized, cats were removed from the chamber and gassed down with isoflurane via a mask and the catheters were placed. Once catheter placements were complete, the cats were monitored while recovering from anesthesia. Ten minutes before glucose administration, each group of cats were injected with either vehicle (10 mM NaOAc, pH 4.5), 1 μg/kg of exenatide, 5 μg/kg of exenatide, 1 μg/kg of [Gln^{28}]exenatide, or 5 μg/kg of [Gln^{28}]exenatide subcutaneously in the periscapular area. At time 0 min, 1 g/kg glucose bolus was administered to each cat (2 mL/kg of a 0.5 g glucose/mL water) over 30 s via the cephalic catheter. At times −10, 0, 2, 5, 10, 15, 30, 45, 60, 90, and 120 min after glucose injection, ∼1.5 mL samples of blood were collected into a syringe through the jugular catheter. A drop of the collected blood was immediately analyzed for blood glucose levels using an Alpha TRAK 2 Veterinary Blood Glucose Monitoring kit following the manufacturer's instructions. The remaining blood was aspirated directly into a 3 mL EDTA (purple top) Vacutainer tube with the addition of 0.9 trypsin inhibitor units of aprotinin (Phoenix Pharmaceuticals #RK-APRO). The plasma was separated and stored at −80°C until analysis. Insulin was measured in duplicates using the Mercodia feline insulin ELISA kit according to the manufacturer's instructions. Plasma [Gln^{28}]exenatide was analyzed by LC/MS/MS as described below. Twenty nine of the 30 cats enrolled in this study had fasting blood glucose under 120 mg/dL. One cat with BG of 180 was inadvertently enrolled in the 1 μg/kg exenatide group. This cat showed the lowest insulin area under the curve (AUC) and C_{15min} and the lowest glucose disposal rate of the cats in his group; nevertheless, results of this cat did not influence any conclusion of this study.

2.6. Statistics

Normality of data was assessed using the Shapiro-Wilk test [26,27]. Two versions of ANOVA were used to assess groups of treatments: Welch's ANOVA was used when each set in a group of treatments passed the Shapiro-Wilk test for normality; the Kruskal-Wallis test (nonparametric ANOVA on Ranks) was used otherwise. Pairwise *P*-values using the nonparametric Kolmogorov-Smirnov test were generated for all pairs of treatment sets in a group when any set in the group failed the normality test. All tests were applied in Prism 8.0.

2.7. LC-MS/MS analysis of exenatide and [Gln^{28}]exenatide

2.7.1. Exenatide analysis

Exenatide calibration standard was prepared freshly in cat plasma at nominal concentrations of 0.25 to 100 ng/mL. Quality control (QC) samples were also prepared freshly at 0.75, 5.0, and 80 ng/mL. For sample processing, 50 μL each of the standard QC and study samples was placed in a 2-mL tube. Then, internal standard working solution was added. Acetonitrile was used to precipitate the proteins, and supernatant was obtained, dried, reconstituted, and transferred into a 96-well plate for LC-MS/MS analysis. The LC-MS/MS analysis was carried out with an AB Sciex Triple Quad 6500 coupled with a Shimadzu HPLC system. The chromatographic separation was carried out on a C18, 50 × 2 mm column with a mobile phase gradient. The mass spectrometer was operated in positive ESI mode. The multiple reaction monitoring transition was (m/z) 838.0 → 396.3 for exenatide and (m/z) 843.5 → 396.3 for internal standard ([Gln^{28}]Exenatide-d10).

2.7.2. [Gln^{28}]exenatide analysis

[Gln^{28}]exenatide samples were analyzed as described for exenatide with the following exceptions. The multiple reaction monitoring transition was (m/z) 841.1 → 396.3 for [Gln^{28}]exenatide and (m/z) 1,090.7 → 650.3 for internal standard (bivalirudin).

3. Results

3.1. Pharmacokinetic effects of GLP-1RA peptides

We compared the pharmacokinetics of [Gln^{28}]exenatide and exenatide in cats (Fig. 1, Table 1). Whether administered intravenously or subcutaneously, pharmacokinetic parameters of the 2 peptides are similar except for slightly longer $t_{1/2,\ \beta}$ values for exenatide and a higher dose-adjusted AUC for [Gln^{28}]exenatide; the $t_{1/2,\ \beta}$ of SC exenatide compares favorably with the reported value of 40 min (from Fig. 4 in [12]). Comparing the dose-adjusted AUCs for intravenously and subcutaneously administered peptides gave a SC bioavailability of 52% for exenatide and 93% for [Gln^{28}]exenatide.

3.2. Pharmacodynamic effects of GLP-1RA peptides

To determine the pharmacodynamic similarity of [Gln^{28}]exenatide and exenatide, we compared their acute glucoregulatory effects in healthy DSH cats (n = 6/group), 12 to 48 mo of age and 4.0 to 6.8 kg of BW, using a high-dose IVGTT (1 g/kg IV glucose bolus) [28] (Table 2). Although body condition scores of cats were not assessed, they were of the same breed and similar BW, so results can be compared among groups. We note that a previous study also determined insulin levels in 12- to 48-month-old, nondiabetic cats during a high-dose IVGTT [28]. However, an anti-porcine insulin antibody was used in that study, so the insulin response cannot be directly compared with those obtained here which used the anti-feline insulin antibody.

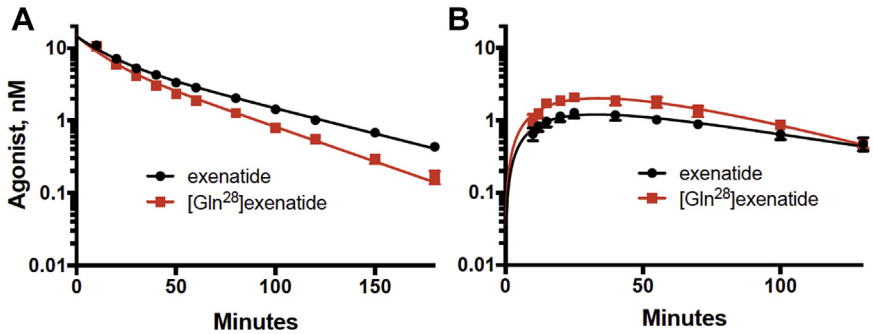


Fig. 1. C vs t plots of plasma exenatide and [Gln^{28}]exenatide in the cat. (A) After intravenous (IV) injections of 10 μg/kg exenatide (●) and [Gln^{28}]exenatide (■) and (B) after subcutaneous (SC) injections of 5 μg/kg exenatide (●) and [Gln^{28}]exenatide (■) during intravenous glucose tolerance test. The points are the average of 6 measurements and error bars are ±SEM (n = 6/group), which are not drawn if bars are shorter than the symbol. The plots were obtained by nonlinear regression using a biexponential models with weighting by $1/SD^2$.

[Gln^{28}]exenatide and exenatide were administered as single SC injections of 1 and 5 μg/kg to cats (n = 6/group) 10 min before the IV bolus of glucose, and blood glucose, plasma insulin, and peptide concentrations were measured over the subsequent 2 h (Fig. 1B). Table 2 summarizes the means ± SD for insulin AUC and C_{max} values for each group, as well as median and interquartile range for cases where normal distribution in the group was suspected. Table 2 also shows cases where comparisons to vehicle have P values < 0.05. Analysis by Welch's ANOVA test and the nonparametric Kruskal-Wallis test showed significance ($P < 0.05$) for the insulin AUC and C_{max} values, respectively. Analysis based on the Kolmogorov-Smirnov test (Table S1) gave pairwise P values < 0.05, showing high-dose [Gln^{28}] exenatide is significantly different from vehicle or low-dose agonists, but not high-dose exenatide.

The results most relevant to the present study are that the insulin AUC and C_{max} values of 5 μg/kg [Gln^{28}]exenatide is significantly higher ($P < 0.05$) than the vehicle control; hence, we can confidently determine a drug-caused effect by 5 μg/kg [Gln^{28}]exenatide using one or both of these parameters.

Table 1
Pharmacokinetic parameters of exenatide and [Gln^{28}]exenatide in the cat.

Pharmacokinetic parameter	Exenatide		[Gln^{28}]exenatide	
	IV[a]	SC[b]	IV[a]	SC[b]
C_{max}, nM	11.0 ± 1.8	1.3 ± 0.4	10.6 ± 1.6	2.1 ± 0.7
k_{β}, h^{-1}	0.96 ± 0.05	0.79 ± 0.13	1.3 ± 0.1	1.1 ± 0.2
$t_{1/2,\beta}$, min	43	52	32	38
$t_{1/2,\alpha}$, min	9.9	12	8.3	13
V_z, L/kg	0.13	0.29	0.16	0.17
AUC_{inf}, nM-h	9.3	2.4	6.9	3.2
Dose-adjusted				
$C_{max,DA}$, nM*kg/nmol	4.6	1.1	4.5	1.8
AUC_{DA}, nM-h*kg/nmol	3.9	2.0	2.9	2.7
Bioavailability (F)		52%		93%

[a] Exenatide or [Gln^{28}]exenatide at 10 μg/kg was injected intravenously, and plasma peptides were measured by LC/MS/MS.
[b] Exenatide or [Gln^{28}]exenatide at 5 μg/kg was injected subcutaneously, and plasma peptides were measured by LC/MS/MS. The k_{β} values are best-fits ±SE, and C_{max} values are mean ± SD.

In contrast, significant differences in glucose AUC were not observed between any drug treatment and vehicle control. The mean glucose AUC_{120min} values did not decrease with 1 μg/kg and only by ~15% with 5 μg/kg of either GLP-1RA peptide. This is not unexpected because small changes in the very large background of high blood glucose in the IVGTT are not easily discernable. Indeed, significant changes were observed in glucose AUC in oral but not IVGTTs in rats and in humans [7,29]. With the GLP-1 RAs, glucose disposal rate followed the control for the initial ~15 min and then increased when the apex of insulin release at 15 min was reached. With 1 μg/kg of either

Table 2
Insulin and glucose measurements in the intravenous glucose tolerance test of cats treated with exenatide (Ex) or [Gln^{28}]exenatide ([Gln^{28}]Ex)[a].

A	Insulin	
Group	$AUC_{120\ min}$, nM-min[b,c]	C_{max}, nM[b,c]
Vehicle	14 ± 7.1	0.22 ± 0.14 (0.19 [0.12, 0.27])
Ex 1 μg/kg	30 ± 5.7 *	0.63 ± 0.26 **
[Gln^{28}]Ex 1 μg/kg	24 ± 7.7 *	0.55 ± 0.36
Ex 5 μg/kg	43 ± 33 *	1.1 ± 1.1 (0.51 [0.38, 2.4])
[Gln^{28}]Ex 5 μg/kg	50 ± 34 *	1.1 ± 0.70 *
B	**Glucose**	
Group	AUC_{120min}, M-min	$t_{1/2}$, min
Vehicle	1.9 ± 0.3	39 ± 14
Ex 1 μg/kg	1.9 ± 0.5	33 ± 10
[Gln^{28}]Ex 1 μg/kg	1.8 ± 0.4	30 ± 9
Ex 5 μg/kg	1.6 ± 0.3	26 ± 11 (20 [18, 38])
[Gln^{28}]Ex 5 μg/kg	1.6 ± 0.2	25 ± 4

[a] Data are presented as mean ± SD. If the distribution of data in a group failed, the Shapiro-Wilk normality test values are also reported as [median (interquartile range)].
[b] Welch's ANOVA for and nonparametric ANOVA (Kruskal-Wallis test) on insulin AUC and insulin C_{max}, respectively, each satisfied $P < 0.05$.
[c] (*) P value vs vehicle satisfies $P < 0.05$; P values were determined by Welch's t-test for differences of means or if a non-normal distribution was suspected in either the treatment or vehicle group, by using the nonparametric Kolomogorov-Smirnov test; (**) P value vs vehicle satisfies $P < 0.01$.

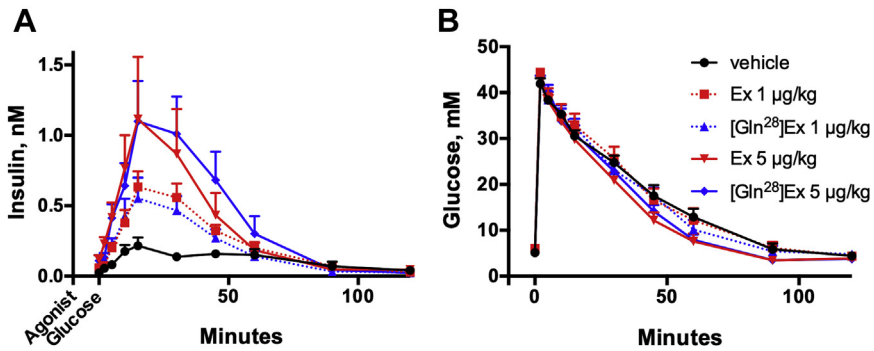


Fig. 2. Acute insulinotropic effects of exenatide (Ex) and [Gln^{28}]exenatide ([Gln^{28}]Ex) during an intravenous glucose tolerance test in cats. (A) Plasma insulin and (B) blood glucose vs time after administration of Ex or [Gln^{28}]Ex 10 min before glucose infusion; agonist doses are 0 (●), 1 µg Ex (■) or [Gln^{28}]Ex (▲)/kg, and 5 µg Ex (▼) or [Gln^{28}]Ex (◆)/kg. Values are the mean + SE.

peptide, the mean glucose disposal $t_{1/2}$ between 15 and 60 min was reduced ~18%, and at 5 µg/kg of either peptide, it was reduced ~33%.

With 1 µg/kg exenatide or [Gln^{28}]exenatide, average insulin AUCs were ~2-fold higher than the average control AUC (Fig. 2). At 5 µg/kg of the GLP-1RAs, the insulin AUC values were ~2- to 3-fold above control for exenatide and ~3.6-fold above control for [Gln^{28}]exenatide. The average insulin C_{15min} values follow a similar response pattern as that for insulin AUCs; the insulin C_{15min} was ~2.5-fold higher than control with 1 µg/kg [Gln^{28}]exenatide and 5-fold higher with 5 µg/kg.

The insulin AUC and C_{15min} values of individual animals proved revealing (Fig. S1). With 5 µg/kg exenatide, 2 cats were high responders (5- and 7-fold above the control average) and 4 showed AUCs and C_{15min} values similar to the average value of the 1 µg dose. With 5 µg/kg [Gln^{28}]exenatide, there was one nonresponder, one very high responder (8-fold above the control average), and 4 moderately high responders who had insulin AUCs and C_{15min} values >3-fold and >3.5-fold, respectively, higher than the average control value. It is confounding that one of the 6 cats treated with 5 µg [Gln^{28}]exenatide had no insulin release yet showed a significant effect on glucose disposal rate. This was not a mishap in drug injection because the plasma [Gln^{28}]exenatide levels were in the expected range. Possibly, the noninsulin-mediated response represents the ability of glucose to regulate its own disposal [30] or a result of GLP-1RA caused decreasing glucagon concentrations.

3.3. Long-acting microsphere-[Gln^{28}]exenatide conjugates

[Gln^{28}]exenatide microspheres with a cyano (CN) modulator in the drug-release linker [23] showed an in vitro $t_{1/2}$ for drug release of about 2,100 h and time for reverse gelation (t_{RG}) for microspheres of about 4,000 h at pH 7.4, 37°C. The [Gln^{28}]exenatide-microsphere conjugate (MS-[Gln^{28}]exenatide) was injected subcutaneously into healthy cats (n = 6/group) at 1.2 and 4.7 µmol/cat (~0.2 and 0.9 µmol/kg, respectively), and plasma [Gln^{28}]exenatide concentration vs time was measured by LC-MS/MS (Fig. 3). The [Gln^{28}]exenatide released from the high dose showed a $t_{1/2,\beta}$ of 43 d, even longer than the 30- to 37-d $t_{1/2,\beta}$ measured in rodents [23]. Over the first 14 d, the plasma peptide from low-dose MS-[Gln^{28}]exenatide showed a linear dose-response relationship with the high-dose preparation. However, after 14 d, the plasma peptide from the lower dose was below the lower limit of detection, and the latter part of the C vs t curve was simulated assuming the $t_{1/2,\beta}$ was the same as the high dose.

Assuming dose linearity, we simulated [23] from these data that monthly injections of microspheres containing 0.23 mg/kg of [Gln^{28}]exenatide would achieve a steady-state C_{min} of ~50 pM [Gln^{28}]exenatide, reflecting the exposure required for antidiabetic efficacy in rodents and humans [9,31]. At this dose, C_{max} would be 80 pM, C_{max}/C_{min} is 1.6, and AUC_{1Mo} is 44 nM*h. For comparison, from Table 1, the commonly used 1 µg/kg BID dose of exenatide would give C_{max} of 250 pM, C_{max}/C_{min} of >250, and AUC_{1Mo} of 27 nM*h.

3.4. Adverse events

Although objectives of this study did not include toxicokinetic determinations, the subjects were observed for signs of injection site reactions and GI disturbances, such as

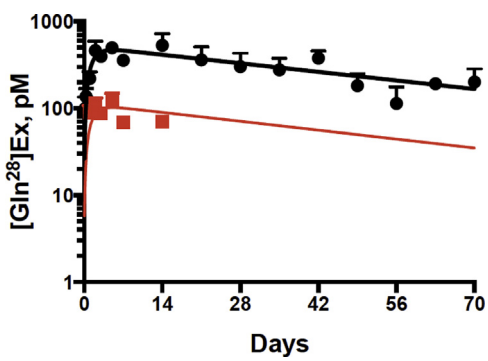


Fig. 3. Plasma [Gln^{28}]exenatide levels after subcutaneous (SC) injection in cats with microsphere conjugates having a CN modulator. After injection of 1.2 µmol [~0.20 ± 0.03 µmol/kg (●)] or 4.6 µmol [~0.90 ± 0.20 µmol/kg (■)] of [Gln^{28}]exenatide/cat, $t_{1/2,\beta}$ was 43 d. The C vs t plot values were obtained by nonlinear regression using a biexponential release and decay model with weighting by $1/SD^2$; error bars are +SEM (n = 6 cats/group). For each cat, a normalized concentration, C_N, was calculated as $C_N = C_m(D_{ave}/D_{inj})$, where C_m is the measured concentration, D_{ave} is the average dose given to all cats, and D_{inj} is the dose injected in the subject cat.

vomiting and diarrhea, that are common adverse events of GLP-1 agonists in humans [32]. Cats dosed with single IV injections of 10 μg/kg of either exenatide or [Gln^{28}]exenatide showed no adverse events over the 3 h period of the pharmacokinetic study. Likewise, no adverse events were observed after SC injection of 1- and 5-μg/kg of the peptides for the 2 h IVGTT.

For longer studies of the microsphere-[Gln^{28}]exenatide, cats were housed as study groups and the total number of vomiting or diarrhea events from cage deposits over the first month were recorded. The events were assessed as accretions in the housing each day, and single vs multiple events were distinguished by the color, consistency, and freshness of spots. In the microsphere-[Gln^{28}]exenatide, 3 vomiting events occurred in the low-dose group in the first 2 d, then 2 on day 10; overall, there were 5 vomiting events in the first month, and there were no events of diarrhea. In the high-dose group, 2 vomiting events occurred in the first 2 d, then one on days 6, 12, and 13; there was a single event of diarrhea on day 6. Over a 1-month period, there were 7 events of vomiting/diarrhea. One cat had a treatment-unrelated urinary tract blockage on day 21 that cleared after catheterization.

Injection site reactions and abdominal discomfort were assessed weekly, and no abnormalities were observed.

3.5. Weight loss and food intake

The BW of each cat was monitored biweekly following a single dose of MS-[Gln^{28}]exenatide. Figure 4 shows individual (Fig. 4A,B) and average (Fig. 4C) BW changes throughout the study. In the low-dose group, the average BW loss was ~5% at 21 d, after which weight increased to normal by ~28 d. Individually, 3 of 6 cats showed a 5% to 14% BW loss over ~1 mo and then gradually returned to normal by 60 d; the other 3 animals did not show a significant BW change throughout the study. In the high-dose group, 5 of 6 cats showed an average BW loss of 11% by 14 d, which remained >10% until day 28 and then slowly returned to normal. Individually, all cats showed a 10% to 14% BW loss over 14 d; 3 recovered by day 28 to 35, 2 remained at >10% BW loss for 56 d, and 1 continued to lose up to 40% BW over the study period. We note that application of Grubbs' test for outliers ($\alpha = 0.05$) to the latter cat identified each time point between 42 and 70 d as outliers.

It was observed that group food intake appeared normal in the low-dose group, but that the high-dose group consistently did not consume their 420 g/d of allotted food.

4. Discussion

The objectives of the present study were as follows. First, to determine whether pharmacokinetic and pharmacodynamic properties of exenatide and [Gln^{28}]exenatide were similar in the cat. If so, the large body of information available for native exenatide could be used to facilitate clinical development of [Gln^{28}]exenatide. Second, to determine whether an MS-[Gln^{28}]exenatide prodrug would provide a drug-delivery system suitable for once-monthly administration in the cat. If so, the system could provide a convenient alternative to BID injections of insulin for treatment of feline diabetes. Finally, we wished to incorporate the information obtained in this work to design a clinical trial in which feline patients are preselected for their response to [Gln^{28}]exenatide. Our hope is that we can preselect diabetic cats that are likely to respond to and benefit from the drug and avoid unnecessarily treating patients that do not.

Previous studies have shown that the pharmacokinetic parameters of exenatide and [Gln^{28}]exenatide are indistinguishable in the mouse and rat [23]. In the cat, the $t_{1/2,\beta}$ of the [Gln^{28}]exenatide was about 20% lower and the SC bioavailability was ~80% higher than exenatide. Otherwise, the pharmacokinetic parameters of exenatide and [Gln^{28}]exenatide are identical in the cat.

The insulinotropic effects of GLP-1RAs are complex but can be briefly summarized as follows [6]. The GLP-1RAs bind to the GLP-1R on pancreatic β-cells and, in the presence of high blood glucose, stimulate insulin secretion and synthesis. The released insulin promotes the physiological uptake of glucose into fat and skeletal muscle, and the subsequent reduction of blood glucose results in a decrease of glucose-dependent GLP-1RA-stimulated insulin release. Hence, the GLP-1RAs operate by a feedback loop that both regulates and is regulated by blood glucose concentration. Acute insulinotropic effects of GLP-1RAs can be monitored during an IVGTT by a) glucose-stimulated insulin secretion and b) glucose disposal.

Exenatide and [Gln^{28}]exenatide are indistinguishable in their insulinotropic effects in rodents [23] and, as shown

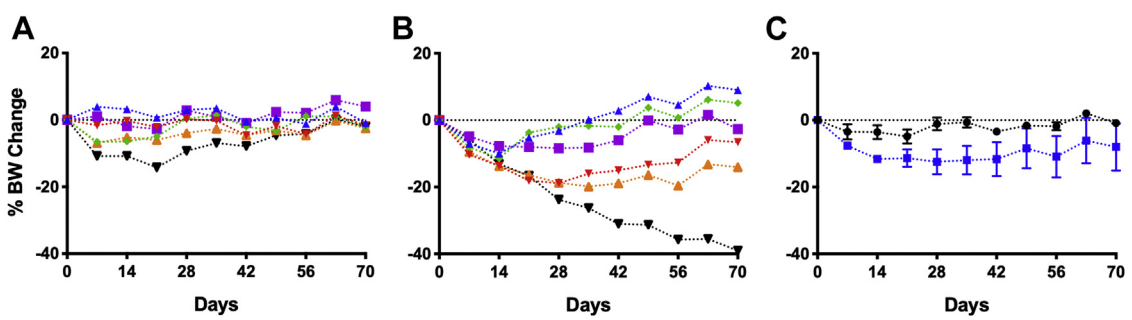


Fig. 4. Weights of cats treated with MS-[Gln^{28}]exenatide. (A) BW changes of individual cats treated with (A) lower dose (4.8 mg peptide/cat) and (B) higher dose (19.3 mg peptide/cat) of MS-[Gln^{28}]exenatide. (C) BW changes of cats treated with MS-[Gln^{28}]exenatide at 4.8 mg peptide/cat (●, N = 6) or 19.3 mg peptide/cat (■, N = 6); the points are averages of measurements ± SE.

here, in the cat. The acute pharmacodynamic effects of SC injections of vehicle control, 1- (0.24 nmol) and 5-μg (1.2 nmol)/kg doses of exenatide and [Gln^{28}]exenatide in the cat were compared during high-dose IVGTTs. Glucose-stimulated insulin secretion, as measured by insulin AUC and C_{15min}, is stimulated by both exenatide and [Gln^{28}] exenatide. At 1 μg/kg of either peptide, the average insulin AUC and C_{15min} values are significantly increased over the control value. These values increase further at 5 μg/kg, suggesting that, in contrast to rodents, insulinotropic effects in the cat may not follow a bell-shape curve. That is, in rodents, the insulinotropic effects of exenatide peaks at a plasma level of ~1 nM, or a dose of ~1 μg/kg [7,23,33], and decrease with higher doses. In contrast, in the cat, average insulin AUC and C_{15min} increases with dose from 1 to 5 μg/kg. At a 5-μg/kg dose, the glucose-stimulated insulin release by [Gln^{28}]exenatide appears somewhat higher and more uniform than that of exenatide. However, because the measured plasma levels of [Gln^{28}]exenatide were higher than exenatide at this dose—likely due to its higher SC bioavailability—it can only be concluded that [Gln^{28}]exenatide is at least as effective as exenatide. The average glucose AUC values in cats were unaffected by the GLP-1RAs studied. However, the glucose disposal rates increased ~50% when cats were treated with the 5 μg/kg dose of either GLP-1RA, similar to the effect of exenatide during IVGTTs in normal and T2D humans [34,35]. In summary, there is no significant difference in insulinotropic effects between [Gln^{28}]exenatide and exenatide in the cat.

We previously reported an MS-[Gln^{28}]exenatide conjugate that is suitable for once-monthly administration in rodents. After SC injection, the depot slowly released the drug and provided [Gln^{28}]exenatide with a plasma $t_{1/2,\beta}$ of 30 to 37 d. The MS-[Gln^{28}]exenatide showed chronic antidiabetic effects in the Zucker diabetic fatty rat that were equivalent to continuous infusion of native exenatide. When injected subcutaneously in cats, the [Gln^{28}]exenatide released from this conjugate showed a plasma $t_{1/2,\beta}$ of ~40 d and a C_{max}/dose of ~550 pM/(μmol/kg). Therapeutic effects of exenatide are generally achieved at plasma levels of about 50- to 70 pM [9,31] which—in view of the equivalent insulinotropic effects of exenatide and [Gln^{28}]exenatide in the cat—serves as a reasonable therapeutic target level for [Gln^{28}]exenatide in the cat. Using pharmacokinetic data reported here, we calculate that monthly doses of MS-[Gln^{28}]exenatide containing only 0.23 mg would be sufficient to maintain a plasma level above 50 pM in the cat. Most studies of exenatide in the cat have used a BID dose of 1 μg/kg. As shown here, at steady state, the peptide released from QMo MS-[Gln^{28}]exenatide would have a similar AUC_{1Mo} as BID exenatide, but a 3-fold lower C_{max}, a much lower C_{max}/C_{min} (1.6 vs > 250), and many fewer peak-trough transitions. From these data, one would predict that the QMo MS-[Gln^{28}] exenatide would be much more tolerable and convenient and possibly more effective than exenatide in the cat.

In humans, gastrointestinal disturbances—in particular nausea, vomiting, and diarrhea—are frequent adverse effects of GLP-1 receptor agonists that are dose-dependent and diminish with prolonged exposure. Considering the very high level of plasma [Gln^{28}]exenatide reached in the high-dose pharmacokinetic experiment—up to 500 pM in plasma, or ~10-fold over the anticipated therapeutic level—there were surprisingly few events of vomiting or diarrhea; however, most animals showed significant weight loss of ~11%, with one outlier losing ~40% BW over 2 mo. In the lower dose experiment, the plasma concentration was closer to the expected therapeutic level and side effects—<1 vomiting event/cat—were considered acceptable. The BW loss in the low-dose experiment—average ~5% loss at 21 d—was similar to that reported with BID exenatide in healthy and diabetic cats [17,36,37]. From these data, along with the low C_{max}/C_{min}, we do not expect the long-acting MS-[Gln^{28}]exenatide to exhibit unexpected toxicities.

Candidate patients for a clinical study of a GLP-1RA in diabetic cats may have unreliable histories as to the time of onset and/or the severity of the disease. A potential problem with treating client-owned cats with a GLP-1RA is that patients may have had longstanding disease and suffer from pancreatic β-cell exhaustion and dysfunction; that is, their pancreatic β-cells may not have sufficient insulin for secretion and hence unable to mount an effective insulinotropic response to GLP-1RAs. Indeed, in a recent report, 6 of 12 client-owned obese cats recruited by public advertisement did not show significant exenatide-stimulated insulin release during a high-dose IVGTT [36]. Because a major benefit of a GLP-1RA is its insulinotropic effect, such medications may have lower utility in feline patients with β-cell dysfunction. In our planned clinical studies of the long-acting MS-[Gln^{28}]exenatide in diabetic cats, we intend to exclude patients who do not show a well-defined insulinotropic effect on treatment with the free, native GLP-1RA drug during a diagnostic IVGTT. That is, cats will be treated with 5 μg/kg [Gln^{28}]exenatide during a screening IVGTT. Cats that do not show an insulin AUC greater than 2 SDs above the vehicle control (ie, >17 nM*min) will be deemed unresponsive and will not be enrolled into the study; by this criterion, 5 of the 6 cats in the current 5 μg/kg [Gln^{28}] exenatide treatment group would have been enrolled in the study. Included patients will be treated with MS-[Gln^{28}] exenatide, aimed at achieving a 50- to 70-pM level of plasma [Gln^{28}]exenatide at steady state.

The premise of this work is that GLP-1RAs will provide a useful therapy for feline diabetes, and a summary of this work is as follows: a) We have shown that pharmacokinetic and pharmacodynamic properties of the GLP-1RAs exenatide and [Gln^{28}]exenatide were similar in the cat; b) We developed an MS-[Gln^{28}]exenatide prodrug, which provides a delivery system suitable for once-monthly administration in the cat; c) We have designed a clinical trial in which diabetic cats are preselected for their response to [Gln^{28}]exenatide. We believe that a once-monthly SC injectable treatment of feline diabetes would be welcomed by veterinarians, client cat owners, and patient cats.

CRediT authorship contribution statement

E.L. Schneider: Conceptualization, Investigation, Project administration, Writing - review & editing. **R. Reid:** Data curation, Formal analysis, Writing - review & editing. **D.G. Parkes:** Methodology, Conceptualization, Writing - review & editing. **T.A. Lutz:** Methodology, Conceptualization,

Writing - review & editing. **G.W. Ashley:** Methodology, Conceptualization, Writing - review & editing. **D.V. Santi:** Methodology, Conceptualization, Writing - original draft.

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All coauthors hold options or units in ProLynx.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.domaniend.2019.07.001.

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