CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

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PHARMACOLOGY REVIEW(S)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY NDA/BLA REVIEW AND EVALUATION

Application number:	206321
Supporting document/s:	1 / 12.20.13 / new NDA
	29 / 07.15.14 / Response to Information Request
Applicant's letter date:	20 December 2013
CDER stamp date:	20 December 2013
Product:	Saxenda™ (liraglutide injection for weight management)
Indication:	An adjunct to a reduced calorie diet and increased physical activity for chronic weight management in overweight adults with at least 1 weight-related co-morbidity or obese adults
Applicant:	Novo Nordisk Inc., Plainsboro, NJ 08536
Review Division:	Endocrinology and Metabolism Products
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1 Executive Summary

1.1 Introduction

Liraglutide, a lipidated glucagon-like peptide-1 (GLP-1) analog, is a GLP-1 receptor (GLP-1R) agonist from Novo Nordisk approved in January 2010 as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus (T2DM) under New Drug Application (NDA) 22341 for Victoza. NDA 206321 from Novo Nordisk seeks marketing approval of Saxenda, 3.0 mg/day liraglutide as an adjunct to a reduced calorie diet and increased physical activity for chronic weight management in overweight adults (initial body mass index (BMI) $\geq$ kg/m^2) with at least 1 weight-related comorbidity (hypertension, prediabetes or T2DM, dyslipidemia, or obstructive sleep apnea) or obese adults (initial BMI $\geq$30 kg/m^2). Saxenda and Victoza both consist of 3 mL of 6 mg/mL liraglutide solution for subcutaneous injection in a laminated rubber disc capped glass cartridge contained in a pen injector, and the only difference between the 2 products is the pen injector. The Victoza FlexPen is capable of delivering up to 1.8 mg liraglutide (0.3 mL) in a single injection, the maximum recommended human dose (MRHD) for the treatment of T2DM, while the Saxenda PDS290 pen injector is capable of delivering up to 3 mg/day liraglutide (0.5 mL dose volume) in a single injection, the only proposed maintenance dose and the MRHD for weight management.

1.2 Brief Discussion of Nonclinical Findings

Pivotal nonclinical studies evaluating the safety of liraglutide were previously reviewed under Victoza NDA 22341 and cross-referenced by Saxenda NDA 206321. Because systemic clearance of subcutaneously injected liraglutide increases with body weight in humans, steady state systemic exposure from 3.0 mg/day liraglutide in obese adults ($AUC_{0\text{-}24h}$ 854 nM*h) was only slightly higher than steady state systemic exposure in healthy adults administered 1.8 mg/day liraglutide ($AUC_{0\text{-}24h}$ 809 nM*h), based on plasma liraglutide AUC comparison. Therefore, human exposure multiples based on systemic exposure for findings in nonclinical safety studies of liraglutide, including carcinogenicity and reproductive and developmental toxicity studies, are similar for 3.0 mg/day liraglutide in obese adults and 1.8 mg/day liraglutide in healthy adults.

Liraglutide (NN2211 or NNC 90-1170) is a lipidated human GLP-1 analog with prolonged pharmacologic activity after subcutaneous bolus injection. Biologic effects of liraglutide are mediated by the GLP-1R, a G-protein coupled receptor coupled to the stimulatory G-protein, Gs. *In vitro*, liraglutide is a selective GLP-1R agonist pharmacologically active at cloned GLP-1Rs from mice, rats, rabbits, pigs, monkeys, and humans. *In vivo*, liraglutide is active in animal models of type 2 diabetes and obesity. While the effects of liraglutide to improve glycemic control are mainly due to blood glucose-dependent enhanced insulin secretion, the effects of liraglutide to reduce food consumption and body weight gain are centrally mediated. Studies evaluating CNS penetration of peripherally administered liraglutide showed liraglutide accesses and activates neurons in brain regions regulating food intake in rats.

Liraglutide was formulated as a solution for subcutaneous injection. Peak and total liraglutide exposure generally increased linearly with dose with no appreciable accumulation in mice, rats, or monkeys and with some accumulation in humans due to its longer elimination half-life (~1.5 fold). Liraglutide was highly plasma protein bound in all species (> 98%). Liraglutide did not readily cross the blood brain barrier and only very low levels were found in the CNS, but the central nervous system is believed to be the site of action for liraglutide's effect to reduce food consumption and body weight gain. Liraglutide circulates as the intact parent drug without forming any major circulating human metabolite. Liraglutide is primarily eliminated by peptidase

metabolism (neutral endopeptidase (NEP) and dipeptidyl peptidase IV (DPP-4)) with further extensive and rapid catabolism of intermediate metabolites prior to excretion in urine, feces, and expired air. Only low levels of liraglutide cross the placenta in rats or rabbits. Intact liraglutide was secreted in milk from rats. Levels of liraglutide in rat milk were ~50% of maternal plasma levels.

Safety and toxicity of liraglutide were evaluated in safety pharmacology studies, single and repeat dose toxicity studies, genetic toxicity studies, 2 year carcinogenicity studies in rats and mice, reproductive and developmental toxicity studies, local tolerance studies, and mechanistic studies of liraglutide-induced thyroid C-cell tumors in rodents. All pivotal nonclinical safety studies were reviewed under Victoza NDA 22341.

In safety pharmacology studies, liraglutide increased heart rate in isolated rabbit hearts and in conscious rats, increased arterial blood pressure and decreased body temperature in rats, induced diuresis in rats, weakly inhibited acetylcholine-induced smooth muscle contraction of isolated guinea pig ileum, and delayed gastric emptying in minipigs. Liraglutide had no effect on the QTc interval in conscious telemetered monkeys.

Liraglutide was well tolerated in chronic repeat dose toxicity studies. Clinical signs of toxicity and reduced food consumption were dose-limiting in rats, while transiently reduced food consumption and decreased body weight gain were dose limiting in rabbits and monkeys. A dose limiting toxicity was not observed in mice.

Liraglutide immunogenicity did not affect exposure to pharmacologically active drug in mice, rats, or monkeys. Liraglutide was not immunogenic in mice or rats, but anti-liraglutide antibodies (anti-drug antibodies, ADAs) occurred in one mid-dose monkey and several high dose monkeys in chronic repeat dose studies. Neutralizing effects of ADAs were not characterized.

Liraglutide toxicity occurred in thyroid (mice and rats), at injection sites (mice, pigs, and monkeys), and it induced a mild anemia (mice, rats, and monkeys). In thyroid, liraglutide caused ultimobranchial cysts and/or C-cell focal hyperplasia, a preneoplastic lesion, in 4 & 13 week mouse studies, a low incidence of inflammatory cell infiltrate in mice in the carcinogenicity study, and C-cell focal hyperplasia and tumors at clinically relevant exposures in carcinogenicity studies in both mice and rats. Injection site reactions were characterized as subacute inflammation and fat necrosis in pigs and inflammation, necrosis, and fibrosis with repeat dosing in monkeys. Although inflammation didn't occur at injection sites in mice, fibrosarcomas in the dorsal skin and subcutis, the body surface used for injection, occurred in high dose male mice in the carcinogenicity study using a liraglutide dosing solution that was 10 times more dilute than the clinical formulation. Local toxicity of the marketed product evaluated after subcutaneous injection in pigs and intramuscular, intravenous, and intra-arterial injection in rabbits showed subcutaneous injection site reactions in pigs attributed to vehicle persisted for 5 days after dosing and intramuscular, intravenous, or intra-arterial injection of liraglutide did not result in severe local toxicity or clinical signs of severe toxicity in rabbits. Mild anemia occurred at clinically relevant exposures in some repeat dose studies in mice, rats, and monkeys. In a 13-week repeat dose study, liraglutide did not cause pancreas inflammation in male or female Zucker Diabetic Fatty (ZDF) rats, a rodent model of insulin resistant type 2 diabetes mellitus.

Liraglutide was not mutagenic or clastogenic *in vitro* with or without metabolic activation in a bacterial reverse mutation assay or a chromosomal aberrations assay in human peripheral blood lymphocytes and it was not clastogenic *in vivo* in erythrocyte micronucleus assays in rats.

Two-year carcinogenicity studies in mice and rats showed liraglutide is a multi-sex, multi-species carcinogen causing thyroid C-cell tumors at clinically relevant exposures in male and female mice and rats and fibrosarcomas on the dorsal skin and subcutis of male mice. Human relevance of drug-induced C-cell tumors in rodents is unknown and a mode of action for liraglutide-induced rodent C-cell tumors has not been established. Mechanistic toxicity studies in wild-type and GLP-1R knockout mice showed liraglutide-induced thyroid C-cell hyperplasia and

increased plasma calcitonin was GLP-1R-dependent, and in normal and hyperplastic thyroid C-cells, liraglutide induced phosphorylation of ribosomal protein S6, but not the REarranged during Transfection (RET) proto-oncogene. Liraglutide-induced proliferative lesions in thyroid C-cells were not fully reversed in mice treated for 9 weeks followed by a 15 week recovery period or in mice treated for 26 weeks followed by a 78 week recovery period.

Liraglutide did not affect fertility of male rats, but in female rats treated with liraglutide from 2 weeks prior to mating through organogenesis, liraglutide increased the number of early embryonic deaths at maternal plasma exposures 11 times systemic exposure in obese humans, based on AUC comparison. In fetal rats, liraglutide caused fetal abnormalities of displaced kidneys, displaced azygous vein, and irregular ossification in the skull and a more complete state of ossification at all doses yielding maternal plasma human exposure multiples $\geq$ 0.8. At the highest dose, mottled liver and minimally kinked ribs occurred at 11-times the human exposure. Major abnormalities of misshapen oropharynx or narrowed larynx occurred at 0.8-times human exposure and umbilical hernia occurred at 0.8 and 3-times human exposure. Pregnant rabbits were treated with liraglutide during organogenesis using doses yielding maternal plasma exposures < 1-times human exposure. Liraglutide decreased fetal weight and increased the incidence of total major fetal abnormalities at all doses, (2.1%, 3.7%, 5.7%, and 7.6% of fetuses and 18%, 30%, 35%, and 32% of litters affected by major abnormalities at 0, 0.01, 0.025, and 0.05 mg/kg liraglutide, respectively). Irregular ossification and/or skeletal abnormalities occurred in the skull and jaw, vertebrae and ribs, sternum, pelvis, and scapula. Visceral abnormalities occurred in blood vessels and gall bladder. In a prenatal and postnatal toxicity study of 0, 0.1, 0.25, or 1 mg/kg liraglutide administered to parental F_0 rats from gestation day 6 through weaning on lactation day 24, liraglutide delayed delivery to day 22 in the majority of treated rats and decreased F_1 generation pup weight at all doses during the lactation period. Bloody scabs and agitated behavior occurred in male rats descended from dams treated with 1 mg/kg/day liraglutide. Group mean body weight from birth to postpartum day 14 trended lower in F_2 generation rats descended from liraglutide-treated rats compared to F_2 generation rats descended from controls, but differences did not reach statistical significance for any group. In a dose range-finding juvenile toxicity study in rats, liraglutide delayed the onset and completion of sexual maturation of male rats, an effect attributed to decreased body weight gain, and liraglutide delayed the completion of sexual development of female rats and reduced the relative weight of ovaries, effects that occurred in the absence of reduced body weight.

1.3 Recommendations

1.3.1 Approvability

Based on prior approval of up to 1.8 mg/day liraglutide for the treatment of type 2 diabetes mellitus, the approval of other long-acting GLP-1 receptor agonists that are known or suspected to induce rodent thyroid C-cell tumors of unknown human relevance, similar steady state systemic exposure to liraglutide in obese adults administered 3.0 mg/day liraglutide compared to healthy adults administered 1.8 mg/day, and no new safety concerns from nonclinical studies for the proposed indication for weight management in obese adults or overweight adults with at least weight-related comorbidity, I recommend approval of up to 3.0 mg/day liraglutide for the proposed weight management indication. The approved label for Victoza and the proposed label for Saxenda both include a boxed warning regarding the unknown human relevance of liraglutide-induced thyroid C-cell tumors in rodents. Review of post-marketing safety information for Victoza by FDA's Division of Pharmacovigilance 1 concluded 6/9 cases of medullary thyroid cancer (MTC) reported to the FDA Adverse Event Reporting System (FAERS) were possibly related to liraglutide treatment. However, further review of Victoza post-marketing case reports of MTC by a thyroid cancer expert in FDA's

Division of Metabolism and Endocrinology Products concluded the relation to liraglutide treatment was unknown and these post-marketing reports of MTC were not an impediment to recommending approval for a large majority of voting members of an 11 September 2014 meeting of the Endocrinologic and Metabolic Drug Advisory Committee evaluating the benefits and risks of 3.0 mg/day liraglutide for the proposed weight management indication.

1.3.2 Additional Non Clinical Recommendations

None.

1.3.3 Labeling

Relevant nonclinical sections in the proposed label are shown below. Recommended deletions are shown by strikethrough text and additions are in red font.

HIGHLIGHTS OF PRESCRIBING INFORMATION

WARNING: RISK OF THYROID C-CELL TUMORS

See full prescribing information for complete boxed warning.

- **Liraglutide causes thyroid C-cell tumors at clinically relevant exposures (b) (4). It is unknown whether (b) (4) Saxenda causes thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans, as human relevance could not be determined by clinical or nonclinical studies (5.1)**.
- **Saxenda is contraindicated in patients with a personal or family history of MTC or in patients with Multiple Endocrine Neoplasia syndrome type 2 (MEN 2) (5.1).**

·····INDICATIONS AND USAGE·····

Saxenda is a glucagon-like peptide-1 (GLP-1) receptor agonist indicated as an adjunct to a reduced calorie diet and increased physical activity for chronic weight management in adult patients with an initial body mass index (BMI) of

- 30 kg/m^2 or greater (obese) (1) or
- 27 kg/m^2 or greater (overweight) in the presence of at least one weight related comorbidity such as hypertension, dysglycemia (prediabetes and type 2 diabetes mellitus), dyslipidemia or obstructive sleep apnea (1)

·····WARNINGS AND PRECAUTIONS·····

- Thyroid C-cell tumors in animals: Counsel patients regarding the risk of medullary thyroid carcinoma and the symptoms of thyroid tumors (5.1).

FULL PRESCRIBING INFORMATION

WARNING: RISK OF THYROID C-CELL TUMORS

Liraglutide causes dose-dependent and treatment-duration-dependent thyroid C-cell tumors at clinically relevant exposures in both genders of rats and mice. It is unknown whether (b) (4) Saxenda causes thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans, as human relevance could not be ruled out by clinical or nonclinical studies. Saxenda is contraindicated in patients with a personal or family history of MTC and in patients with Multiple Endocrine Neoplasia syndrome type 2 (MEN 2). (b) (4)

[see Contraindications (4), Warnings and Precautions (5.1) (b) (4)

5.1 Risk of Thyroid C-cell Tumors

Liraglutide causes dose-dependent and treatment-duration-dependent thyroid C-cell tumors (adenomas and/or carcinomas) at clinically relevant exposures in both genders of rats and mice *[see Nonclinical Toxicology (13.1)]*. Malignant thyroid C-cell carcinomas were detected in rats and mice. (b) (4) . It is unknown whether Saxenda will cause thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans, as the human relevance of liraglutide-induced rodent thyroid C-cell tumors (b) (4) (b) (4) determined (b) (4)

5.5 Renal Impairment

(b) (4)

8.1 Pregnancy

Pregnancy Category (b) (4) X.

Risk Summary

There are no adequate and well-controlled studies of Saxenda in pregnant women. Saxenda should not be used during pregnancy. If a patient wishes to become pregnant, or pregnancy occurs, treatment with Saxenda should be discontinued.

Clinical Considerations

A minimum weight gain, and no weight loss, is recommended for all pregnant women, including those who are already overweight or obese, due to the necessary weight gain that occurs in maternal tissues during pregnancy.

Animal Data

Liraglutide has been shown to be teratogenic in rats at or above (b) (4) 0.8-times (b) (4) systemic exposures in obese humans resulting from the maximum recommended human dose (MRHD) of 3.0 mg/day based on plasma area under the time-concentration curve (AUC) comparison. Liraglutide has been shown to cause reduced growth and increased total major abnormalities in rabbits at systemic exposures below (b) (4) exposure in obese humans at the MRHD based on plasma AUC comparison.

Female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide beginning 2 weeks before mating through gestation day 17 had estimated systemic exposures (b) (4) 0.8- (b) (4) 3-, and (b) (4) 11-times the (b) (4) exposure in obese humans at the MRHD based on plasma AUC comparison. The number of early embryonic deaths in the 1 mg/kg/day group increased slightly. Fetal abnormalities and variations in kidneys and blood vessels, irregular ossification of the skull, and a more complete state of ossification occurred at all doses. Mottled liver and minimally kinked ribs occurred at the highest dose. The incidence of fetal malformations in liraglutide-treated groups exceeding concurrent and historical controls were misshapen oropharynx and/or narrowed opening into larynx at 0.1 mg/kg/day and umbilical hernia at 0.1 and 0.25 mg/kg/day.

Pregnant rabbits given subcutaneous doses of 0.01, 0.025 and 0.05 mg/kg/day liraglutide from gestation day 6 through day 18 inclusive, had estimated systemic exposures less than (b) (4) -exposure in obese humans at the MRHD of 3.0 mg/day at all doses, based on plasma AUC comparison. Liraglutide decreased fetal weight and dose-dependently increased the incidence of total major fetal abnormalities at all doses. The incidence of malformations exceeded concurrent and historical controls at 0.01 mg/kg/day (kidneys, scapula), ≥ 0.01 mg/kg/day (eyes, forelimb), 0.025 mg/kg/day (brain, tail and sacral vertebrae, major blood vessels and heart, umbilicus), ≥ 0.025 mg/kg/day (sternum) and at 0.05 mg/kg/day (parietal bones, major blood vessels). Irregular ossification and/or skeletal abnormalities occurred in the skull and jaw, vertebrae and ribs, sternum, pelvis, tail, and scapula; and dose-dependent minor skeletal variations were observed. Visceral abnormalities occurred in blood vessels, lung, liver, and esophagus. Bilobed or bifurcated gallbladder was seen in all treatment groups, but not in the control group.

In pregnant female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide from gestation day 6 through weaning or termination of nursing on lactation day 24, estimated systemic exposures were (b) (4) 0.8-, (b) (4) 3-, and (b) (4) 11-times ~~human~~ exposure in obese humans at the MRHD of 3.0 mg/day, based on plasma AUC comparison. A slight delay in parturition was observed in the majority of treated rats. Group mean body weight of neonatal rats from liraglutide-treated dams was lower than neonatal rats from control group dams. Bloody scabs and agitated behavior occurred in male rats descended from dams treated with 1 mg/kg/day liraglutide. Group mean body weight from birth to postpartum day 14 trended lower in F_2 generation rats descended from liraglutide-treated rats compared to F_2 generation rats descended from controls, but differences did not reach statistical significance for any group.

8.3 Nursing Mothers

It is not known whether liraglutide is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for tumorigenicity shown for liraglutide in animal studies, a decision should be made whether to discontinue nursing or to discontinue Saxenda, taking into account the importance of the drug to the mother. In lactating rats, liraglutide was excreted unchanged in milk at concentrations approximately 50% of maternal plasma concentrations.

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

A 104-week carcinogenicity study was conducted in male and female CD-1 mice at doses of 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide administered by bolus subcutaneous injection yielding systemic exposures (b) (4) 0.2-, 2- (b) (4) 10- and (b) (4) 43-times the exposure in obese humans (b) (4) respectively, at the maximum recommended human dose (MRHD) of 3.0 mg/day based on plasma AUC comparison. A dose-related increase in benign thyroid C-cell adenomas was seen in the 1.0 and the 3.0 mg/kg/day groups with incidences of 13% and 19% in males

and 6% and 20% in females, respectively. C-cell adenomas did not occur in control groups or 0.03 and 0.2 mg/kg/day groups. Treatment-related malignant C-cell carcinomas occurred in 3% of females in the 3.0 mg/kg/day group. Thyroid C-cell tumors are rare findings during carcinogenicity testing in mice. A treatment-related increase in fibrosarcomas was seen on the dorsal skin and subcutis, the body surface used for drug injection, in males in the 3 mg/kg/day group. These fibrosarcomas were attributed to the high local concentration of drug near the injection site. The liraglutide concentration in the clinical formulation (6 mg/mL) is 10-times higher than the concentration in the formulation used to administer 3 mg/kg/day liraglutide to mice in the carcinogenicity study (0.6 mg/mL).

A 104-week carcinogenicity study was conducted in male and female Sprague Dawley rats at doses of 0.075, 0.25 and 0.75 mg/kg/day liraglutide administered by bolus subcutaneous injection with exposures (b) (4) 0.5-, (b) (4) 2- and (b) (4) -times the exposure in obese humans- (b) (4) respectively, resulting from the MRHD based on plasma AUC comparison. A treatment-related increase in benign thyroid C-cell adenomas was seen in males in 0.25 and 0.75 mg/kg/day liraglutide groups with incidences of 12%, 16%, 42%, and 46% and in all female liraglutide-treated groups with incidences of 10%, 27%, 33%, and 56% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. A treatment-related increase in malignant thyroid C-cell carcinomas was observed in all male liraglutide-treated groups with incidences of 2%, 8%, 6%, and 14% and in females at 0.25 and 0.75 mg/kg/day with incidences of 0%, 0%, 4%, and 6% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. Thyroid C-cell carcinomas are rare findings during carcinogenicity testing in rats.

Studies in mice demonstrated that (b) (4) liraglutide-induced C-cell proliferation was dependent on the GLP-1 receptor (b) (4) -and that liraglutide did not cause activation of the REarranged during Transfection (RET) proto-oncogene in thyroid C-cells.

Human relevance of thyroid C-cell tumors in mice and rats is unknown and could not be determined by clinical studies or nonclinical studies *[see Boxed Warning and Warnings and Precautions (5.1)]*.

Liraglutide was negative with and without metabolic activation in the Ames test for mutagenicity and in a human peripheral blood lymphocyte chromosome aberration test for clastogenicity. Liraglutide was negative in repeat-dose *in vivo* micronucleus tests in rats.

In rat fertility studies using subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide, males were treated for 4 weeks prior to and throughout mating and females were treated 2 weeks prior to and throughout mating until gestation day 17. No direct adverse effects on male fertility was observed at doses up to 1.0 mg/kg/day, a high dose yielding an estimated systemic exposure (b) (4) 11-times the (b) (4) -exposure in obese humans at the MRHD, based on plasma AUC comparison. In female rats, an increase in early embryonic deaths occurred at 1.0 mg/kg/day. Reduced body weight gain and food consumption were observed in females at the 1.0 mg/kg/day dose.

2 Drug Information

2.1 Drug

CAS Registry Number: 0204656-20-2
Generic Name: liraglutide

Code Name: NNC 90-1170, NNC 0090-0000-1170, NN2211, glipacyl
Chemical Name: $Arg^{34}Lys^{26}$-(N-ε-(γ-Glu-(N-α-hexadecanoyl)))-GLP-1[7-37]
Molecular Formula/Molecular Weight: $C_{172}H_{265}N_{43}O_{51}$ / 3751.2 Daltons
Structure or Biochemical Description:

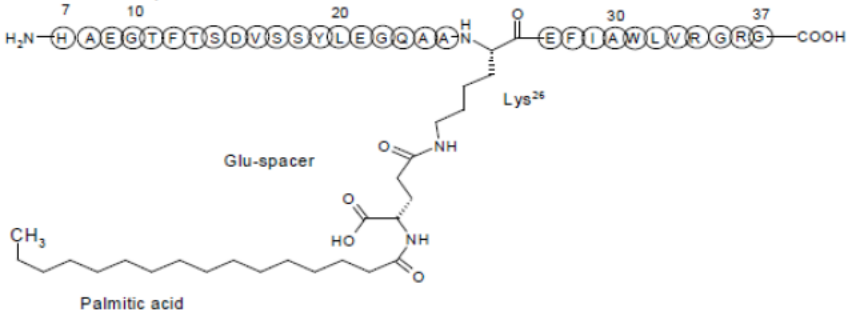

Figure 1 Molecular structure of liraglutide
One-letter amino acid codes are used in this figure.

[SD1 Module 2.6 Nonclinical Written and Tabulated Summaries Introduction P6]

Pharmacologic Class: glucagon-like peptide 1 (GLP-1) receptor agonist

2.2 Relevant INDs, NDAs, BLAs and DMFs

IND 61040: liraglutide for the treatment of type 2 diabetes mellitus (opened in October 2000, sponsored by Novo Nordisk)
IND 73206: liraglutide for the treatment of obesity (opened September 2008, sponsored by Novo Nordisk)
NDA 22341: Victoza® (liraglutide for injection), up to 1.8 mg/day liraglutide for the treatment of type 2 diabetes mellitus (approved January 2010, from Novo Nordisk)

DMF (b) (4) : (b) (4)
DMF (b) (4) :

2.3 Drug Formulation

Liraglutide drug product is an aqueous, clear, colorless or almost colorless solution of liraglutide in a glass cartridge provided in a pre-filled pen injector. The glass cartridge containing 3 mL of 6.0 mg/mL liraglutide solution is closed with a laminated rubber disc consisting of a (b) (4) The composition of 6.0 mg/mL liraglutide solution is shown in Table 1 (below). The liraglutide cartridge in Saxenda is identical to the cartridge in the approved product Victoza.

Table 1 Composition of liraglutide 6.0 mg/ml

Name of ingredients	Quantity per ml	Function	Reference to standards
Active substance			
Liraglutide	6.0 mg	Active drug substance	Novo Nordisk A/S
Excipients			
Disodium phosphate, dihydrate	1.42 mg	(b) (4)	Ph. Eur., USP
Phenol	5.5 mg[1]		Ph. Eur., USP, JP
Propylene glycol	14.0 mg		Ph. Eur., USP, JP
(b) (4)		(b) (4)	Ph. Eur., USP, JP
			Ph. Eur., USP, JP
Water for Injections		(b) (4)	Ph. Eur., USP, JP

1. (b) (4)

[SDN 1 Description and Composition of the Drug Product P3]

For both Saxenda and Victoza, the cartridge containing 3 mL of 6 mg/mL liraglutide is provided in a pre-filled pen injector, and the only difference between the drug products is the pen injectors. The Victoza FlexPen is capable of delivering up to 1.8 mg liraglutide (0.3 mL dose volume) in a single injection, the maximum recommended human dose (MRHD) for the treatment of type 2 diabetes mellitus (T2DM), while the Saxenda PDS290 pen injector is capable of delivering up to 3 mg/day liraglutide (0.5 mL dose volume) in a single injection, the only proposed maintenance dose and MRHD for weight management. Because initiating liraglutide therapy requires dose escalation over the course of several weeks to improve tolerability, the PDS290 pen injector is capable of delivering doses of 0.6 mg, 1.2 mg, 1.8 mg, 2.4 mg, or 3 mg liraglutide per injection (corresponding to dose volumes of 0.1, 0.2, 0.3, 0.4, and 0.5 mL, respectively). Doses $\leq$2.4 mg/day liraglutide are intended for stepwise dose escalation while 3.0 mg/day is the proposed maintenance dose for the weight management indication. Figure 2 (below) shows the components of the PDS290 pen-injector.

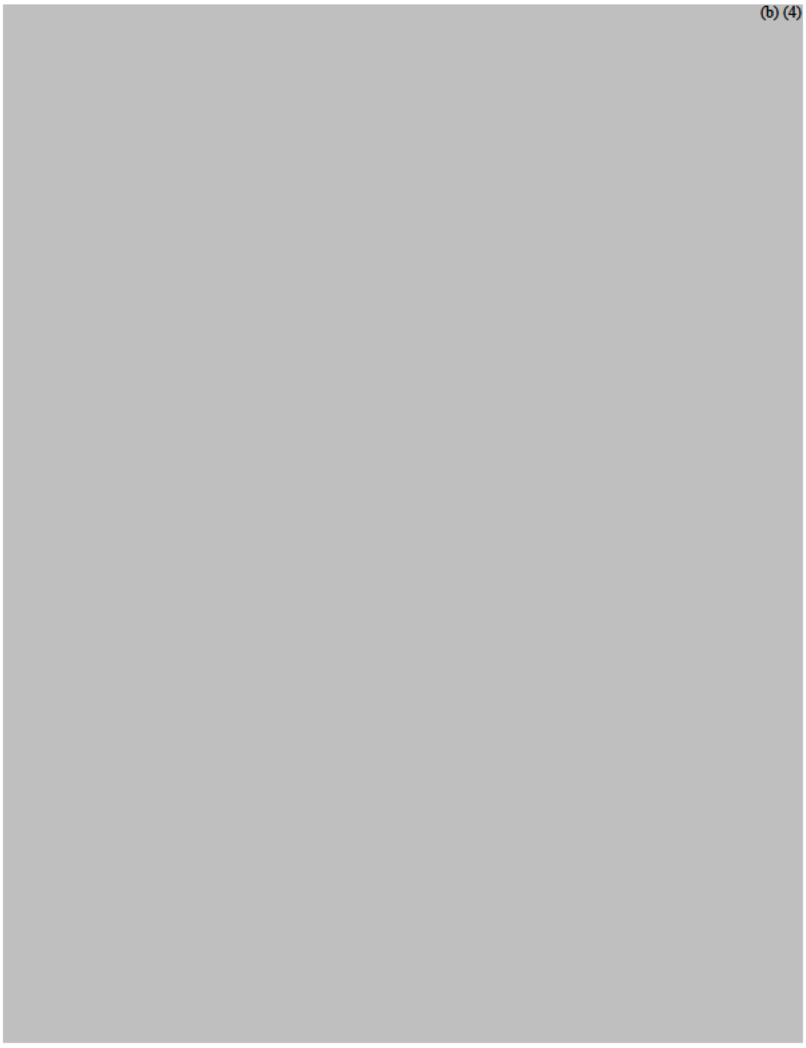

Figure 2 Exploded view of the PDS290 pen-injector for liraglutide
[SDN 1 Description an Composition of the Drug Product P4]

PDS290 pen injectors containing 18 mg / 3 mL liraglutide solution will be packaged in sample packs containing 1 or 3 pieces and trade packs containing 3 or 5 pieces. The shelf life is (b) (4) months at 2 – 8C, the same as Victoza®.

The current marketed formulation was used in single dose tolerance studies in rabbits and pigs, a 104-week carcinogenicity study in mice, some clinical pharmacology trials (applicant didn't specify which trials), phase 2 study NN8022-1807 in obese subjects, and 3 phase 3 clinical studies for weight management.

2.4 Comments on Novel Excipients

There are no novel excipients.

2.5 Comments on Impurities/Degradants of Concern

Qualification of Impurities and Degradation Products

Toxicity of liraglutide in an old formulation (drug substance from (b) (4) and new formulation (drug substance from (b) (4) was evaluated in a 4-

week repeat subcutaneous dose study in Sprague Dawley rats (10/sex/dose) using doses of 0 (vehicle) or 1 mg/kg/day liraglutide (old and new formulations). The new formulation had undergone forced degradation by storing it at 37C for 2 months prior to the study. There were no substantive differences in toxicity between old and new formulations of liraglutide.

Table 22 (below) shows impurity levels in the drug product compared to batches of liraglutide used in repeat dose toxicity studies. There are no new concerns about impurities in liraglutide drug substance.

Table 22 Impurity levels non-clinical studies and in drug product to be marketed based on in-use acceptance criteria

	Study ID	NN980186	NN201109	NN980186 NN990284	NN980183 NN980189	NN980184 NN990191	NN200240	NN200241	NN204229	NN205092	Drug Product[a)]
	Species	Rabbit	Rat	Rat	Rat	Monkey	Rat	Monkey	Mouse	Rat	
Impurity	Liraglutide, mg/kg/day	0.03	1.0	1.0	1.0	5.0	0.75	5.0	3.0	1.0	0.06 [b)]
Sum of liraglutide related impurities	Level, (%)	(b) (4)									
	Exposure, µg/kg										
Other (b) (4) liraglutide related impurities	Level, (%)										
	Exposure, µg/kg										
Liraglutide related impurities A	Level, (%)										
	Exposure. µg/kg										
Liraglutide related impurities B	Level, (%)										
	Exposure µg/kg										
Liraglutide related impurities C	Level, (%)										
	Exposure µg/kg										
Other (b) (4) liraglutide related impurities	Level, (%)										
	Exposure µg/kg										
HMWP	Level, (%)										
	Exposure µg/kg										

a) Maximum exposure based on in-use acceptance criteria. b) Assuming 3.0 mg maximum daily dose and minimum 50 kg BW. N.A.: not analyzed . N.D.: not determined

Toxicity studies referenced in Table 22 (above) are:

NN980186: NNC 90-1170: Preliminary segment I/II subcutaneous reproduction study in rats (incorrect species in table)
NN201109: NNC 90-1170: Pre and post natal study in rats (subcutaneous administration)
NN980186: NNC 90-1170: Preliminary segment I/II subcutaneous reproduction study in rats
NN990284: NNC 90-1170: Main segment I/II subcutaneous reproduction study in rats
NN980183: NNC 90-1170: 28 day subcutaneous toxicity study in rats
NN980189: NNC 90-1170: 13 week subcutaneous toxicity study in rats with recovery period
NN980184: NNC 90-1170: 28 day subcutaneous toxicity study in cynomolgus monkeys
NN990191: NNC 90-1170: 13 week subcutaneous toxicity study in the cynomolgus monkey with a recovery period
NN200240: NNC 90-1170: 104 week carcinogenicity study in rats with subcutaneous administration
NN200241: NNC 90-1170: 52 week subcutaneous toxicity study in the cynomolgus monkey with a 4 week recovery period
NN204229: NNC 90-1170: 104 week carcinogenicity study in mice with subcutaneous administration
NN205092: NNC 90-1170: 4 week toxicity study in rats with subcutaneous administration (bridging study)

[SD1 Toxicology Written Summary P55]

Leachables

Table 2 shows leachables from the container closure system detected after storage of liraglutide-filled cartridges for up to (b) (4) months at 2 – 8C followed by (b) (4) days at 30C. Five organic and 3 inorganic leachables were identified.

Table 2 Concentration of leachables in 3 ml Penfill® cartridges detected after 36 months at 2-8 °C followed by 32 days at 30 °C and maximal clinical exposure to leachables

Compound	CAS number	Maximal concentration (µg/mL)	Max. clinical s.c. exposure (µg/person/day)[a]
(b) (4)			

[SDN 1 Nonclinical Overview P31]

The applicant believes exposure to leachables does not pose any safety risk and the container closure system is suitable for the intended use. The total daily dose of (b) (4) ((b) (4) mcg/day) is (b) (4)-fold lower than the (b) (4) mg/day residual solvent limit for (b) (4) specified in the Guidance for Industry titled "Q3C Impurities: Residual Solvents (December 1997)" and "Q3C — Tables and List (November 2003)". (b) (4) are inactive ingredients present at much higher concentrations in approved products (FDA's website titled "Inactive Ingredients Search for Approved Drug Products" at http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm). The average (b) (4) level in human blood was (b) (4) mg/L ((b) (4) mcg/mL, ranging from (b) (4) mcg/mL) ((b) (4) and a daily dose of (b) (4) mcg (b) (4) is approximately (b) (4) of the total (b) (4)

Safety Assessment for (b) (4) *and* (b) (4)

(b) (4) ((b) (4) and (b) (4) ((b) (4) are drug product impurities believed to be products of a reaction between (b) (4) To limit the production of (b) (4) to below the threshold of toxicological concern (TTC, 1.5 mcg/day for known or potential genotoxic or carcinogenic impurities) during clinical studies evaluating the use of liraglutide for weight management under IND 73,206, the applicant proposed limiting the storage of drug product to (b) (4) days at (b) (4) C (March 2008 IND 73,206 Nonclinical Review from Dr. David Carlson). At this time during development, the maximum proposed dose for weight management was 3.0 mg/day liraglutide. The limit of (b) (4) mcg/day (b) (4) was below the TTC and below the threshold for degradants in new drug products as per the ICH Guidance for Industry titled "Q3B(R2) Impurities in New Drug Products (July 2006)".

In the chemistry review for Victoza NDA 22341 supporting the marketing of up to 1.8 mg/day liraglutide for the treatment of T2DM (December 2008 Chemistry Review by Dr. Joseph Leginus), (b) (4) were identified as leachables from the (b) (4) that occurred at levels up to (b) (4) mcg/mL yielding a maximum daily exposure of (b) (4) mcg/day (b) (4) below the TTC.

In NDA 206321 supporting the marketing of 3.0 mg/day liraglutide (0.5 mL of 6.0 mg/mL liraglutide) for weight management, the applicant reported the maximum concentration of (b) (4) (b) (4) as leachables from the container closure system (b) (4) months at 2 – 8C

followed by (b) (4) days at 30C) was (b) (4) mcg/mL yielding a maximum clinical exposure of (b) (4) mcg/day, above the TTC. In patients taking up to 1.8 mg/day liraglutide for the treatment of T2DM, the total daily dose of (b) (4) mcg/day (b) (4) exceeds the TTC. The applicant justifies the safety of up to (b) (4) mcg/day (b) (4) based on:

(b) (4)

(b) (4)

(b) (4) and (b) (4) were not genotoxic based on *in silico* analysis predicting (b) (4) and (b) (4) would be negative in bacterial mutagenicity assays, *in silico* analysis predicting (b) (4) and (b) (4) would be positive in mammalian cell clastogenicity assays *in vitro*, and based on negative results *in vivo* in mouse micronucleus assays for both (b) (4) and (b) (4) Evaluation of genetic toxicity by quantitative structure activity relationship (QSAR) analysis performed by the applicant predicted (b) (4) and (b) (4) would not be mutagenic in bacteria, but both would be clastogenic *in vitro* in mammalian cells (Appendix 1). Evaluation of genetic toxicity by QSAR analysis performed by Chemical Informatics Group at CDER predicted (b) (4) and (b) (4) would be negative in bacteria reverse mutation assays, but both would be clastogenic *in vitro* in mammalian cells (Appendix 2). (b) (4) and (b) (4) were both negative *in vivo* in mouse bone marrow erythrocyte assays. The oral LD_{50} of (b) (4) in mice was (b) (4) mg/kg (WHO. Concise International Chemical Assessment Document (b) (4) . (b) (4) was mildly nephrotoxic at high doses in female rats (b) (4) (b) (4) was not nephrotoxic in rats. The oral LD_{50} of (b) (4) in mice was (b) (4) mg/kg (TOXNET website http://toxnet.nlm.nih.gov/). (b) (4) (b) (4) In the absence of genotoxicity, it is unlikely that a total dose of (b) (4) mcg/day (b) (4) and (b) (4) combined would result in any substantive toxicity.

2.6 Proposed Clinical Population and Dosing Regimen

The clinical program for Saxenda evaluated the use of 3.0 mg/day liraglutide as an adjunct to a reduced-calorie diet and increased physical activity for chronic weight management in adult patients with an initial body mass index (BMI) of:

- 30 kg/m^2 or greater (obese), or
- 27 kg/m^2 or greater (overweight) in the presence of at least 1 weight-related comorbidity such as dysglycemia (pre-diabetes and type 2 diabetes mellitus), hypertension, dyslipidemia, or obstructive sleep apnea.

Liraglutide can be subcutaneously injected in the abdomen, thigh, or upper arm once a day any time of day without regard to meals. To improve gastrointestinal tolerability, the starting dose is 0.6 mg/day liraglutide for at least 1 week, and the dose should be escalated at a maximum rate of 0.6 mg/day/week liraglutide to the maintenance dose of 3.0 mg/day liraglutide. The effectiveness of liraglutide for weight loss should be evaluated after at least 12 weeks of treatment with 3.0 mg/day liraglutide.

2.7 Regulatory Background

New Drug Application (NDA) 22341 for Victoza®, liraglutide injection (6 mg/mL) as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus (T2DM), was approved in January 2010. The use of the same formulation of liraglutide for weight management was investigated under IND 73,206 opened in September 2008. During a combined pre-IND / End-of-Phase 2 meeting in April 2008, the Agency and Novo Nordisk agreed that toxicological assessments conducted to support a diabetes indication would be sufficient to support a weight management indication. In May 2012, the applicant requested guidance from CDER's Controlled Substances Staff (CSS) regarding the need for abuse potential studies, and in September 2012, the Agency agreed studies assessing the abuse potential of liraglutide would not be required to support an NDA for a weight management indication, based on nonclinical information submitted to Victoza® NDA 22341 and the clinical and nonclinical rationale provided by the applicant. A pre-NDA meeting between the FDA and Novo Nordisk concerning an application for the use of liraglutide for weight management was held in September 2013, but there were no nonclinical issues discussed at the meeting, and the Agency agreed with the applicant's proposal to cross reference pertinent nonclinical information in Victoza NDA 22341.

NDA 206321 applying for marketing approval of Saxenda, 6 mg/mL liraglutide injection indicated as an adjunct to a reduced calorie diet and increased physical activity for chronic weight management in overweight adults (BMI $\geq$27 kg/m^2) with at least 1 weight-related comorbidity (hypertension, prediabetes or type 2 diabetes mellitus, dyslipidemia, or obstructive sleep apnea) or obese adults (BMI $\geq$ 30 kg/m^2) was submitted as a type 505(b)(1) application and it was received on 20 December 2013. To support NDA 206321, the applicant cross-referenced information in modules 2, 3, 4, and 5 in Victoza NDA 22341 and all subsequent supplements including nonclinical information in modules 2 and 4. In addition to cross-referenced nonclinical information from NDA 22341, the applicant submitted reports of nonclinical studies evaluating the mode of action for liraglutide's effects on appetite, food consumption, and body weight (Table 1, below) and additional toxicology studies to support the weight management indication (Table 2, below). Reports for 3 studies fulfilling nonclinical post-marketing requirements for Victoza were submitted and reviewed under NDA 22341: study 210145 titled "Liraglutide (NNC 90-1170): 104 Week study in CD-1 mice with 26 week subcutaneous administration" for PMR 1583-3, study LoSi100801 titled "Liraglutide (NNC 0090-

0000-1170): A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes" for PMR 1583-4, and study 209306 titled "Liraglutide and exenatide: Toxicity study by subcutaneous administration to GLP1r knock-out and wild-type CD-1 mice for 13 weeks" for PMR 1583-5.

Table 1 Overview of non-clinical pharmacology studies conducted to support the weight management indication

Study type	Duration	Route of administration	Species	Location
Activation of brain neurons				
cFos expression	Acute	s.c.	Rat	4.2.1.1, Kdhl090408
mRNA expression in hypothalamus and brainstem	28 days	s.c.	Rat	4.2.1.1, KLYK130304
Brain penetration and localisation of liragltuide				
Fluorescence technique	4 days	s.c.	Mouse	4.2.1.1, AASC120603
Immuno-histochemsitry and *in situ* ligand binding	Acute-	*In vitro*	Monkey	4.2.1.1, GUS2012-059-NN
Assessment of specific brain areas				
Ablation of area postrema	21 days	s.c.	Rat	4.2.1.1, KLYK130302
Lesion of the paraventricular nucleus	14 days	s.c.	Rat	4.2.1.1, KLYK130303
Ablation of afferent vagal nerve	14 days	s.c.	Rat	4.2.1.1, KLYK130301

[SDN 1 Nonclinical Written and Tabulated Summaries Introduction P8]

Table 2 Additional studies conducted after submission of the Victoza® NDA 22-341

Study type and duration	Duration	Route of administration	Species	Location
(b) (4)				
Local tolerance				
Early development drug product and final drug product	2-5 days	s.c,	Pig	4.2.3.6, NN208224
Final drug product	2-5 days	i.m, i.v., i.a.	Rabbit	4.2.3.6, NN208428
Other toxicity studies				
Effect on liraglutide on human receptors – functional assay on GLP-2, secretin, GHRH, and VPAC2 (VIP2) receptors	NA	*In vitro*	Human	4.2.3.7.3, CEREP 15582
Acute effects of liraglutide on plasma calcitonin after a single subcutaneous administration	1 day	s.c.	GLP-1-receptor knock-out mice and CD-1 mice	4.2.3.7.3, 209188
Acute effects of exenatide on plasma calcitonin after a single subcutaneous administration	1 day	s.c.	GLP-1-receptor knock-out mice and CD-1 mice	4.2.3.7.3, 205207
In-situ ligand binding on the GLP-1R in thyroid tissue *in vitro*	NA	*In vitro*	Rat and human	4.2.3.7.3, CGo081003
3-month study on the effect on C-cell proliferation, and rearranged-during-transfection (RET) proto-oncogene activation and GLP-1R density[§]	3 month	s,.c.	GLP-1-receptor knock-out mice and CD-1 mice	4.2.3.7.3, 209306
104 week study in CD-1 mice with 26 week subcutaneous administration[§]	2 years	s.c.	CD-1 mice	4.2.3.7.3, 210145
Histopathology Extension to (b) (4) Study ID. 506326/203262 (87 week study)	87 weeks.	s.c.	Cynomolgus monkey	4.2.3.7.3, 208304
Statistical analysis of the correlation between initial calcitonin change and focal C-cell hyperplasia and adenoma scores from study NN204163/ NN204310 and NN20511	NA	NA	Rat	4.2.3.7.3, 409.SqA.2058
Other				
3-month study to assess pancreatitis in an animal model of T2D[§]	3 months	s.c.	ZDF Rat	4.2.3.7.7, LoSi100801
Evaluation from pancreas from high dose animals from toxicity Studies in cynomolgus macaques against NCI diagnostic criteria of PanIN	4- 87 weeks	s.c.	Cynomolgus monkey	4.3, PanIN pathology Study report

[§] Conducted as FDA Post Marketing Requirement study for Victoza®. NA; Not applicable.

[SDN 1 Nonclinical Written and Tabulated Summaries Introduction P9-10]

Nonclinical information submitted in NDA 206321 included a nonclinical overview (module 2.4), nonclinical written and tabulated summaries (module 2.6), 7 primary pharmacology study reports (module 4.2.1.1), 5 analytical methods and validation reports (module 4.2.2.1), 1 reproductive and developmental toxicity study report (module 4.2.3.5.4), 2 local tolerance study reports (module 4.2.3.6), 9 mechanistic toxicity study reports (module 4.2.3.7.3), and 1 other toxicity study report of a pancreas safety study (module 4.2.3.7.7).

During review of Saxenda NDA 206321, the Division requested the applicant provide additional information concerning potential genetic toxicity of (b) (4) and (b) (4) leachables in the drug product that form after container closure. The applicant provided the requested information in a 15 July 2014 amendment to the NDA (supporting document 29).

NDA 206321 included a Proposed Pediatric Study Request (module 1.9.4) and proposed REMS (module 1.16). (b) (4) The applicant requests a deferral of clinical studies of liraglutide in children with obesity until it is approved for weight management in adults. Clinical study NN8022-3967 evaluating the pharmacokinetics / pharmacodynamics of liraglutide in obese adolescents 12 to <18 years old is ongoing in Germany. The goal of the proposed REMS is to inform providers about the potential risk of medullary thyroid carcinoma and the risk of acute pancreatitis associated with Saxenda and to inform providers that Saxenda and Victoza both contain the same active ingredient, liraglutide, and they should not be used together.

A meeting of the Endocrinologic and Metabolic Advisory Committee evaluating the efficacy and safety of liraglutide was held on 11 September 2014. A large majority of the Committee considered the overall benefit-risk assessment of 3 mg/day liraglutide favorable to support its approval for the proposed weight management indication.

3 Studies Submitted

3.1 Studies Reviewed

Pharmacology (Primary)

- The access of liraglutide to rodent brain (report AASC120603)
- Mapping of GLP-1 receptor expression in the non-human primate brain (report GUS2012-059-NN)
- Acute effects of liraglutide on cFos expression in the rat brain (report Kdhl090408)
- Liraglutide effects on body weight: The role of vagal afferents (report KLYK130301)
- The role of area postrema in mediating liraglutide effects on food intake and body weight (report KLYK130302)
- The chronic effect of liraglutide in paraventricular nucleus lesioned animals (report KLYK130303)
- The central effects of chronic liraglutide treatment in HE fed DIO rats (report KLYK130304)

Pharmacology (Secondary)

- In vitro pharmacology: Functional assays – study of liraglutide (report CEREP 15582)

Pharmacokinetics (Analytical Methods)

- Validation of NNC 0090-0000-1170 in mouse plasma using Watson LIMS (report 210282)

- Validation of an IRMA for determination of calcitonin in mouse EDTA plasma (report 210285)
- Qualification of NNC 0090-1170 in ZDF rat K2EDTA plasma (report 211027)
- Validation of an ELISA for determination of NNC 0090-0000-1170 in rat EDTA plasma (report 211283)
- Long-term stability of NNC 0090-0000-1170 in rat EDTA plasma (report 211334)

Genetic Toxicity

- (b) (4) Induction of micronuclei in the bone marrow of treated mice (report 210060)
- (b) (4) Induction of micronuclei in the bone marrow of treated mice (report 210064)

(b) (4)

Special Toxicity (Local Tolerance)

- Local toxicity 2 and 5 days after subcutaneous injection in the pig (report NN208224)
- Local tolerance study in rabbits (report NN208428)

Special Toxicity (Pancreas Toxicity)

- Histopathology extension to (b) (4) study no. 506326 (NNC 90-1170 (liraglutide) investigative subcutaneous toxicity study in cynomolgus monkeys (report 208304)
- Liraglutide: Evaluation of pancreas from high dose animals from toxicity studies in cynomolgus macaques against diagnostic criteria for PanIN

Special Toxicity (Mechanistic Carcinogenicity Studies)

- NNC 0113-0000-0000: Study of the acute effects on plasma calcitonin after a single subcutaneous administration in fasted GLP-1 receptor knock-out mice and CD-1 mice (report 205207)
- NNC 0090-0000-1170: Study of the acute effects on plasma calcitonin after a single subcutaneous administration in fasted GLP-1 receptor knock-out mice and CD-1 mice (report 209188)
- Statistical analysis of the correlation between initial calcitonin change and focal C-cell hyperplasia and adenoma scores from study NN204163, NN204310, and NN205119 (report 409 SqA 2058)
- In-situ ligand binding on the GLP-1 receptor in rat and human thyroid (report CGo081003)
- Rodent C-cell findings: Assessment of human relevance

The following studies submitted in Saxenda NDA 206321 were previously reviewed under Victoza NDA 22341

Special Toxicity (Pancreas Toxicity)

- A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes (report LoSi100801)

Special Toxicity (Mechanistic Carcinogenicity Studies)

- Liraglutide and exenatide toxicity study by subcutaneous administration to GLP1r knock-out and wild-type CD-1 mice for 13 weeks (report 209306)

- 104 Week study in CD-1 mice with 26 week subcutaneous administration (report 210145)

3.2 Studies Not Reviewed

None

3.3 Previous Reviews Referenced

Victoza® NDA 22341 Nonclinical Reviews

***July 10, 2009 Review,* pivotal safety and toxicology studies reviewed were:**

Safety Pharmacology

- 990263 / Modified Irwin screen test in the mouse
- 980091 / Evaluation of NNC 90-1170 on respiration in conscious rats205242 / Effect on hERG tail current recorded from stably transfected HEK293 cells
- 980422 / Effects on QT interval and MAP duration in isolated perfused rabbit hearts
- 990264 / Effect on cardiovascular function in the telemetered rat
- 980092 / Evaluation of NNC 90-1170 on cardiovascular function in conscious cynomolgus monkeys
- 990262 / Effect on the renal function in the rat
- 201207 / Effect on the isolated ileum in the guinea pig

Single Dose Toxicity

- 980178 / Acute subcutaneous toxicity test in mice
- 980175 / Acute subcutaneous toxicity test in rats

Repeat Dose Toxicity

- 204082 / 13 week toxicity study in mice with subcutaneous administration
- 980189 / 13 week subcutaneous toxicity study in rats with recovery period
- 200239 / 26 week subcutaneous toxicity study in rats
- 990191 / 13 week subcutaneous toxicity study in the cynomolgus monkey with a recovery period
- 200241 / 52 week subcutaneous toxicity study in cynomolgus monkeys with a 4 week recovery period

Genetic Toxicity

- 980191 / NNC 90-1170 glipacyl reverse mutation in four histidine-requiring strains of Salmonella typhimurium and two tryptophan-requiring strains of Escherichia coli – Ames
- 203114 / Induction of chromosome aberrations in cultured human peripheral lymphocytes
- 980192 / Induction of micronuclei in the bone marrow of treated rats
- 990072 / Assessment of micronucleus frequencies on microscope slide preparations from rats

Carcinogenicity

- 204229 / 104 week carcinogenicity study in mice with subcutaneous administration (review Appendix A)

- 200240 / 104 week carcinogenicity study in rats with subcutaneous administration (review Appendix B)

Reproductive and Developmental Toxicity

- 990284 / Main segment I/II subcutaneous reproduction study in rats
- 990055 / Developmental toxicity study in rabbits
- 201109 / Pre and post natal study in rats (subcutaneous administration)

Local Tolerance

- 204291 / Local toxicity of 3 Phase 3 formulations with pH 7.7, 7.9, and 8.15 two and 5 days after subcutaneous injections in pigs

Special Toxicity
Mechanistic Studies: Rodent Thyroid C-cell tumors (Review Appendix C)

Impurities

- 205092 / 4 week toxicity study in rats with subcutaneous administration (bridging study)

December 16, 2011 Review, studies submitted to fulfill nonclinical post-marketing requirements

PMR 1583-4

- LoSi100801 / A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes

PMR 1583-5

- 209306 / Liraglutide and exenatide: Toxicity study by subcutaneous administration to GLP-1r knock-out and wild-type CD-1 mice for 13 weeks

November 16, 2013 Review, studies submitted to fulfill nonclinical post-marketing requirements

PMR 1583-3

- 210145 / Liraglutide (NNC 90-1170): 104 week study in CD-1 mice with 26 weeks subcutaneous administration

4 Pharmacology

Brief Summary

Primary Pharmacodynamics

Glucagon-like peptide-1 (GLP-1) is a 30- or 31-amino acid peptide primarily secreted from epithelial L-cells in the distal small intestine and colon in response to ingesting food and GLP-1 is a neurotransmitter produced in preproglucagon neurons in the brain, spinal cord, and olfactory bulb. The 30-amino acid amidated form and the 31-amino acid glycine extended form of GLP-1 are equipotent, but in humans, GLP-1(7-36)amide is the predominant circulating active GLP-1. The effects of GLP-1 are mediated by a single, Gs-coupled, family B 7 transmembrane G-protein coupled receptor, the GLP-1 receptor (GLP-1R). GLP-1R is widely expressed

occurring in the pancreas (alpha, beta, and delta cells), peripheral and central nervous systems, heart, kidney, lung (surfactant-secreting type II pneumocytes), and stomach (parietal cells). The primary effects of liraglutide to improve glycemic by increasing glucose-dependent insulin secretion and decreasing glucose-dependent glucagon secretion are mediated by GLP-1Rs in the pancreas while the primary effect of liraglutide to reduce body weight gain by reducing food consumption is likely mediated by GLP-1Rs in the brain.

Liraglutide is a lipidated GLP-1 analog with prolonged pharmacologic activity after subcutaneous injection due to delayed absorption and elimination. Resistance to metabolism by DPP-4 or NEP, reduced renal excretion, and delayed absorption resulting from self-association of the attached lipid prolong its activity. Liraglutide is highly protein bound to albumin further increasing its resistance to DPP-4 or NEP-mediated hydrolysis and reducing renal excretion. *In vitro*, liraglutide was a GLP-1R agonist pharmacologically active in all species used in nonclinical studies. Liraglutide increased cAMP accumulation in cells expressing cloned recombinant mouse, rat, rabbit, pig, monkey and human GLP-1Rs (EC_{50} 5 – 60 nM). The potency of liraglutide was reduced in the presence of albumin, consistent with protein binding diminishing the concentration of free liraglutide.

Pharmacodynamics of Liraglutide for the Treatment of Type 2 Diabetes Mellitus

Primary pharmacology nonclinical studies of liraglutide evaluating its efficacy for the treatment of type 2 diabetes mellitus were reviewed under Victoza NDA 22341. Liraglutide improved glycemic control by: 1) increasing glucose-dependent insulin secretion, 2) decreasing glucose-dependent glucagon secretion, and 3) delayed gastric emptying (although this effect may be less important due to the rapid development of tachyphylaxis). *Ex vivo*, nanomolar concentrations of liraglutide dose-dependently increased glucose-dependent insulin secretion mouse pancreatic islets, inhibited cytokine-induced or free fatty acid-induced apoptosis and stimulated proliferation of pancreatic beta cells from rats. *In vivo*, liraglutide was active in animal models of type 2 diabetes and obesity. Liraglutide did not cause hypoglycemia in normal or diabetic animals and it inhibited the development of diabetes in prediabetic rats. Liraglutide did not lower blood glucose in non-diabetic, insulin resistant Zucker obese rats. Liraglutide improved glycemic control in mouse, rat, and pig models of type 2 diabetes, slowed the onset of diabetes in pre-diabetic rats, and reversed diabetes symptoms in diabetic sand rats.

Pharmacodynamics of Liraglutide for Weight Management

GLP-1 is an important satiety hormone in rodents and humans. The effects of GLP-1 that lower food intake are centrally mediated, but both peripherally and centrally secreted GLP-1 may modulate food consumption because GLP-1 crosses the blood-brain barrier. GLP-1 is secreted from intestinal L cell and released from the brain stem nucleus of the solitary tract and GLP-1Rs are localized in brain areas regulating food intake including the hind brain area postrema and nucleus of the solitary tract, the hypothalamic arcuate nucleus and paraventricular nucleus, and the vagus nerve.

The following primary pharmacology effects from nonclinical studies supporting the use of liraglutide for weight management were previously reviewed under Victoza NDA 22341. In repeat dose toxicity studies, liraglutide transiently reduced food consumption in both sexes of CD-1 mice, Sprague Dawley rats, and cynomolgus monkeys, but consistent effects of liraglutide to lower body weight gain and body weight were observed in male rats and monkeys, but not in male mice or female mice, rats, or monkeys. Liraglutide-related decreased body weight gain in rats was due to reduced food consumption, altered food preference, and unaltered energy expenditure even as body weight decreased.

Liraglutide decreased food consumption and body weight gain in animal models of obesity. In a 7-day repeat dose study of 0.2 mg/kg liraglutide administered twice a day to normal Sprague Dawley rats, liraglutide reduced body weight gain and food consumption without

affecting the respiratory exchange ratio, energy expenditure, bone mineral content, bone area, or bone mineral density. Liraglutide (0.15 mg/kg) had no effect on energy expenditure or substrate oxidation in normal fasted Sprague Dawley rats. In diet-induced obese Sprague Dawley rats, subcutaneous injection of 0.2 or 0.3 mg/kg liraglutide twice a day for 4 weeks decreased subcutaneous fat (inguinal, epididymal, mesenteric, and perirenal), decreased plasma triglycerides, elevated free fatty acids, and in an oral glucose tolerance test, it decreased plasma glucose exposure ($AUC_{0\text{-}180min}$) and increased insulin secretion. In Wistar rats with monosodium glutamate-induced deficits in hypothalamic arcuate nucleus GLP-1R signaling, subcutaneous injection of 0.2 mg/kg liraglutide twice a day for 7 days decreased food consumption, water consumption, body weight, and adiposity. In Zucker diabetic fatty (ZDF) rats, single subcutaneous doses of 0.007 to 6.6 mg/kg liraglutide dose-dependently decreased food consumption and blood glucose. In ZDF rats subcutaneously administered 0.03 or 0.15 mg/kg liraglutide twice a day for 6 weeks, 0.15 mg/kg liraglutide decreased food intake and improved glycemic control, but paradoxically increased body weight. In candy-fed diet-induced obese Sprague Dawley rats, a model of type 2 diabetes, 0.2 mg/kg liraglutide administered subcutaneously twice a day shifted food preference to normal chow, decreased food consumption, decreased body weight, reversed body weight gain, and reversed body fat gain while maintaining energy expenditure, but liraglutide had no beneficial effect on glycemic control or pancreatic beta cell mass.

Single daily subcutaneous injections of 0.003 – 0.007 mg/kg liraglutide for 7 weeks decreased food consumption, by decreasing the number, duration, and size of meals, and decreased body weight of Gottingen minipigs, a model of extreme hyperphagia. Liraglutide's effects in minipigs were reversed after treatment was stopped. Food consumption in non-diabetic glucose intolerant rhesus monkeys with middle-age onset obesity was dose-dependently decreased over a 16 day period with 0.01 or 0.03 mg/kg/day liraglutide with recovery to baseline when treatment was stopped. Liraglutide (0.1 mg/kg liraglutide twice a day) normalized olanzapine-induced increased food consumption, body weight, subcutaneous fat deposits, fasting plasma glucose, and plasma cholesterol in female Sprague Dawley rats. In obese, insulin-resistant, diabetic, leptin-deficient *ob/ob* mice, single subcutaneous doses of 0.03 to 1.0 mg/kg liraglutide dose-dependently decreased food intake, body weight gain, and blood glucose.

In NDA 206321, the applicant submitted additional reports of pharmacology studies evaluating the access of liraglutide to the central nervous system and regions of the CNS that may mediate the effects of liraglutide to lower food consumption (Table 1, below).

Table 1 Overview of non-clinical pharmacology studies conducted to support the weight management indication

Study type	Duration	Route of administration	Species	Location
Activation of brain neurons				
cFos expression	Acute	s.c.	Rat	4.2.1.1, Kdh1090408
mRNA expression in hypothalamus and brainstem	28 days	s.c.	Rat	4.2.1.1, KLYK130304
Brain penetration and localisation of liragltuide				
Fluorescence technique	4 days	s.c.	Mouse	4.2.1.1, AASC120603
Immuno-histochemsitry and *in situ* ligand binding	Acute-	*In vitro*	Monkey	4.2.1.1, GUS2012-059-NN
Assessment of specific brain areas				
Ablation of area postrema	21 days	s.c.	Rat	4.2.1.1, KLYK130302
Lesion of the paraventricular nucleus	14 days	s.c.	Rat	4.2.1.1, KLYK130303
Ablation of afferent vagal nerve	14 days	s.c.	Rat	4.2.1.1, KLYK130301

[SDN1 module 2.6.1 Pharmacology Written and Tabulated Summary Introduction P8]

Peripherally administered liraglutide occurs in circumventricular brain regions and adjacent brain regions in mice and rats. The distribution of liraglutide in brains was determined in male wild-type (WT) C57 mice and male GLP-1R deficient (GLP-1R KO) mice subcutaneously injected with a single dose of 0.45 mg/kg fluorescent-labeled liraglutide or up to 0.45 mg/kg fluorescent liraglutide twice a day for 4 days, a dose of fluorescent liraglutide that reduced body weight gain in diet-induced obese C57 mice, but not in lean C57 mice or GLP-1R KO mice. GLP-1R-indpendent liraglutide-related fluorescence was detected in circumventricular organs (choroid plexus and zona externa of the median eminence) of both WT and GLP-1R KO mice, but localization of fluorescent-liraglutide-related material to specific circumventricular regions (area postrema, zona interna of the median eminence, subfornical organ, and organum vasculosum of the lamina terminus) and regions of the hypothalamus (paraventricular nucleus, arcuate nucleus, dorsomedial hypothalamic nucleus, and supraoptic nucleus) was GLP-1R-depenent because it occurred in WT mice, but not in GLP-1R KO mice. The presence of liraglutide-related material in brain parenchyma from mice after peripheral administration was confirmed by capillary depletion of brain homogenate from mice administered a single subcutaneous dose of 0.2 or 0.4 mg/kg [^{3}H-hexadecanoyl]liraglutide. Radiolabeled liraglutide-related material in brains from mice and rats administered a single subcutaneous dose of [^{3}H-hexadecanoyl]liraglutide was localized to the median eminence (mice and rats), arcuate nucleus (mice and rats), the paraventricular nucleus of the hypothalamus (rats) and the area postrema (mice). In brain from rats subcutaneously administered a single dose of fluorescent liraglutide, fluorescent liraglutide-related material was co-localized with cocaine and amphetamine related transcript (CART) immunoreactivity in some neurons in the arcuate nucleus in the hypothalamus.

In brains from cynomolgus monkeys, GLP-1Rs were localized to regions involved in regulation of food intake. GLP-1R distribution in brains from cynomolgus monkeys was determined by in situ hybridization (GLP-1R mRNA), immunohistochemical analysis (GLP-1R protein), and in situ ligand binding (GLP-1R binding activity). GLP-1R mRNA, protein, and ligand binding activity generally colocalized with high levels of GLP-1R expression in specific sites of the brainstem and hypothalamus. In the brainstem, the highest levels of GLP-1R mRNA, protein, and ligand binding activity occurred in the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus, and GLP-1R was also detected in lateral parts of the

reticular formation, lateral parabrachial nucleus, dorsal raphe, the A1/C1 area, and the gigantocellular nucleus. In the hypothalamus, the highest levels of GLP-1R transcript, protein, and ligand binding activity occurred in cell bodies and fibers of the paraventricular nucleus and dorsomedial nucleus. Low levels of GLP-1R transcript, protein, and ligand binding activity occurred in the paraventricular nucleus of the thalamus. High levels of GLP-1R protein occurred in the central and medial nuclei of the amygdala and the bed nucleus of the stria terminalis with lower levels in the nucleus accumbens. GLP-1R transcript and radioligand binding activity occurred in the substantia nigra and ventral tegmental area, but GLP-1R protein was not detected by immunohistochemical analysis. GLP-1R transcript, protein, or radioligand binding activity was not detected in the cerebral cortex (frontal, occipital, temporal, or parietal).

Activated brain areas in fasted or fed male Sprague Dawley rats were identified by measuring cFos expression levels 4 hours after a single subcutaneous dose of 0 (vehicle) or 0.1 mg/kg liraglutide, a dose that reduced food consumption 2.25 to 4 hours after dosing. Liraglutide increased the number of activated neurons in the area postrema of fed and fasted rats and increased the proportion of activated dopaminergic and/or adrenergic neurons in the area postrema of fed rats, but not in fasted rats. In fasted rats, some neurons in the area postrema activated by liraglutide were not adrenergic or dopaminergic. In the nucleus of the solitary tract, liraglutide activated non-GLP-1 neurons and reduced activation of GLP-1 neurons in fed rats. Liraglutide decreased the number and the proportion of activated cocaine- and amphetamine related transcript (CART) neurons in the arcuate nucleus of fed rats, but not in fasted rats. Liraglutide increased the number of activated neurons in the lateral parabrachial nucleus of fed and fasted rats and in the central amygdala of fed rats, but liraglutide had no effect on the number of activated neurons in the central amygdala of fasted rats or in the paraventricular nucleus of fed or fasted rats.

In 8 month old diet-induced obese male Sprague Dawley rats, 0.1 mg/kg liraglutide subcutaneously administered twice a day for 28 days reduced food consumption, body weight, and fat mass and increased mRNA encoding the anorexigenic peptide CART in the arcuate nucleus and paraventricular nucleus without increasing mRNAs encoding orexigenic peptides neuropeptide Y (NPY) or agouti-related peptide (AgRP).

The role of afferent vagal nerves and specific brain areas that may mediate liraglutide-induced reduced food consumption and body weight gain were evaluated by comparing the effects of 0.2 mg/kg liraglutide injected twice a day for 14 days in rats with lesions of afferent vagal nerves (surgical subdiaphragmatic vagal deafferentiation), brainstem area postrema (by needle aspiration), and hypothalamic paraventricular nucleus (electrolytic lesions). Lesions of the afferent vagal nerves, the brain stem area postrema, or the hypothalamic arcuate nucleus or paraventricular nucleus did not mitigate liraglutide-induced decreased food consumption or decreased body weight gain. A recently published study shows the lateral parabrachial nucleus mediates anorexic effects of GLP-1R agonists, at least in part (Richard et al, Endocrinology. 2014 Aug 13:en20141248. [Epub ahead of print], PMID: 25116706)

Mechanism of action

Liraglutide is an injectable, lipidated analog of human GLP-1(7-37) that is a GLP-1R agonist active at nanomolar concentrations *in vitro*. *In vivo*, prolonged GLP-1R agonist activity of liraglutide is attributed to slower absorption from subcutaneous injection sites due to self-association of liraglutide's lipid moiety, resistance to inactivation by peptidases (DPP-4 and NEP), and reduced renal clearance. Liraglutide activates the GLP-1R, a membrane-bound 7-transmembrane receptor coupled to the adenylyl cyclase stimulating protein, Gs. *In vivo*, subcutaneously injected liraglutide accesses regions of the brain important for regulating food consumption in rodents. In the arcuate nucleus of obese rats, liraglutide increased mRNA encoding the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) and liraglutide was colocalized on CART neurons. Liraglutide activated neurons in rat brain areas

that regulate food consumption including the area postrema and lateral parabrachial nucleus. Although liraglutide reduces food consumption and weight gain in animal models of obesity, specific brain areas mediating the anorexic effects of liraglutide were not identified.

Drug activity related to proposed indication for weight management

Liraglutide reduces food consumption resulting in decreased body weight gain or body weight, primarily due to a reduction in body fat. *In vivo* in animal models of obesity, liraglutide decreased gastric emptying, reduced food intake, altered food preference, and reduced body fat, but energy expenditure was unaffected. In humans, liraglutide increased feelings of satiety and fullness and lowered feelings of hunger and prospective food consumption leading to lower calorie intake and body weight loss. Liraglutide-induced body weight loss was primarily due to a reduction in body fat.

Secondary Pharmacodynamics

In vitro pharmacology screening studies showed liraglutide is GLP-1R selective and it did not interact with off-target sites including ion channels, enzymes, or other G-protein coupled receptors, including the human glucagon receptor, and liraglutide was devoid of agonist activity (EC_{50} > 10 μM) or antagonist activity (IC_{50} >10 μM) at the following cloned human receptors: glucagon-like peptide 2 (GLP-2), secretin, growth hormone releasing hormone (GHRH), or vasoactive intestinal peptide receptor 2 (VIP_2 or $VPAC_2$).

Safety Pharmacology

Liraglutide was evaluated in neurobehavioral, pulmonary, cardiovascular, renal, and gastrointestinal safety pharmacology studies and these studies were reviewed under Victoza NDA 22341. Major findings from these studies were 1.43 μM liraglutide weakly and reversibly inhibited acetylcholine-induced smooth muscle contraction in isolated strips of guinea pig ileum, 1.43 μM liraglutide increased heart rate 6% in isolated rabbit heart, a single subcutaneous dose of 0.2 and 2 mg/kg increased arterial blood pressure, increased heart rate, and decreased body temperature for up to 24 hours after dosing in conscious telemetered Sprague Dawley rats (effects on blood pressure are probably rodent-specific), and liraglutide had a diuretic effect in water-loaded Sprague Dawley rats reducing urine osmolarity and specific gravity and increasing urine volume 2 – 6 hours after dosing with 0.02, 0.2, or 2 mg/kg liraglutide with proteinuria occurring at the highest dose during the 6 – 24 hour collection period.

Pharmacodynamic Drug Interactions

Studies evaluating pharmacodynamic interactions between liraglutide and glipizide, pioglitazone, or olanzapine were previously reviewed under Victoza NDA 22341. *Ex vivo* in isolated perfused rat pancreata, liraglutide dose-dependently increased insulin secretion elicited by glipizide. *In vivo*, combined treatment with liraglutide and pioglitazone for 6 weeks improved glycemic control in male diabetic ZDF rats better than either treatment alone. In male diabetic ZDF rats treated for 6 weeks, liraglutide improved glycemic control and decreased plasma triglycerides and cholesterol to a greater extent than either treatment alone. Liraglutide normalized olanzapine-induced increased food consumption, body weight, subcutaneous fat deposits, fasting plasma glucose, and plasma cholesterol in female Sprague Dawley rats.

4.1 Primary Pharmacology

Study Title: The access of liraglutide to rodent brain (report AASC120603, non-GLP)

Key Study Findings

- Comparing localization of fluorescent liraglutide related material in brain of WT mice and GLP-1 receptor (GLP-1R) deficient mice peripherally administered fluorescent liraglutide showed:
  - fluorescent liraglutide related material localization to specific circumventricular organs (area postrema, zona interna of the median eminence, subfornical organ, and organum vasculosum of lamina terminus) was GLP-1R-dependent while localization to the choroid plexus and zona externa of the median eminence was GLP-1R-independent.
  - localization of fluorescent liraglutide related material to specific regions of the hypothalamus was (paraventricular hypothalamic nucleus, arcuate nucleus, dorsomedial hypothalamic nucleus and supraoptic nucleus) was GLP-1R-dependent.
- Localization of radiolabeled liraglutide-related material in brains of mice and rats peripherally administered [^{3}H-hexadecanoyl]liraglutide, showed liraglutide-related radiolabeled material was localized in median eminence (mice and rats), arcuate nucleus (mice and rats), and paraventricular nucleus (rats) of the hypothalamus and in the area postrema of mice.
- In brain from rats peripherally administered fluorescent liraglutide, fluorescent liraglutide-related material was co-localized with CART immunoreactivity in some neurons in the arcuate nucleus in the hypothalamus in rats.
- Capillary depletion of brain homogenate from mice administered a single subcutaneous dose of 0.2 or 0.4 mg/kg [^{3}H-hexadecanoyl]liraglutide confirmed liraglutide radiolabeled material crossed the blood brain barrier in mice.
- Study deficiencies were:
  - Liraglutide does not consistently reduce food intake or body weight in most laboratory strains of mice maintained on a normal rodent diet.
  - Specificity of the rabbit anti-CART polyclonal antibody (Ca7-OVA, (b) (4) was not demonstrated.

Localization of liraglutide to specific brain regions was evaluated after peripheral administration of fluorescent-liraglutide in male C57bl WT or GLP-1R-deficient (GLP-1R-/-) mice or radiolabeled liraglutide in male C57bl mice and male Sprague Dawley rats.

Fluorescent and Radiolabeled GLP-1 Receptor Ligands

Fluorescent GLP-1R ligands were synthesized by conjugating VivoTag-S® 750 Near IR Fluorochrome label ((b) (4) to liraglutide to yield Vivotag750-liraglutide or by conjugating Alexa Fluor® 594 C5-maleimide (b) (4)) to liraglutide or exendin(9-39) to yield Alexa594-liraglutide or Alexa594-exendin(9-39), respectively. The structure of VivoTag750-liraglutide is shown in Figure 17, below.

Figure 17 Structure of VivoTag-750 liraglutide

[P33]

In vitro, the affinity of VivoTag750-liraglutide for the GLP-1R (species not disclosed) in the presence of high or low albumin concentration (albumin concentrations not disclosed) was similar to liraglutide (Table 2, below).

Table 2 IC50 values for VivoTag-750 liraglutide for GLP-1R
The receptor binding was measured in assays containing high and low albumin concentration respectively.

Compound	GLP-1R binding (high albumin) [IC50 (nM)]	GLP-1R binding (low albumin) [IC50 (nM)]
liraglutide (mean value)	13.9	0.5
VivoTag750-liraglutide	19.1	0.7

[P33]

The applicant contends Figure 18 shows Vivotag750-liraglutide (10 mL/kg, unknown dose because unknown concentration of Vivotag750-liraglutide in dose solution) decreased body weight gain of diet-induced obese C57 mice, but not lean C57 mice or GLP-1R-deficient (GLP-1R-/-) mice (5 / group), but experimetal details (Appendix B of the study report, lacks detail about Vivotag750 liraglutide dose, injection procedure) are not sufficient to evaluate the reliability of results from this portion of the study.

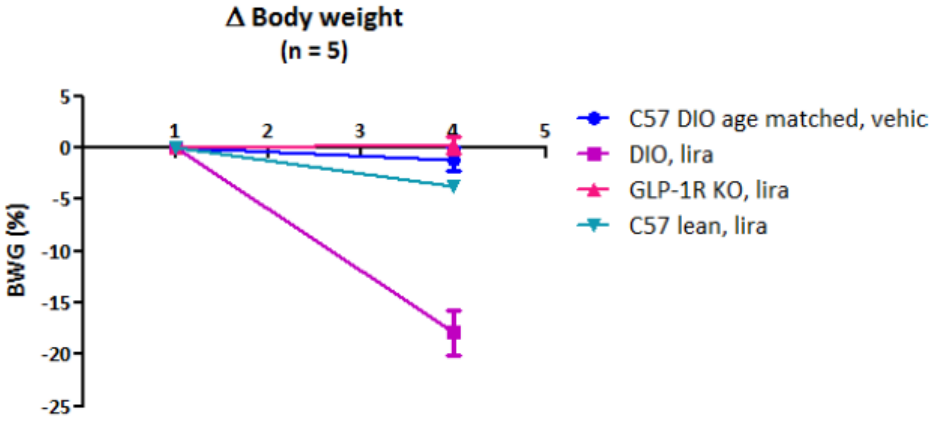

Figure 18 Bodyweight gain following administration with VivoTag750-liraglutide
Bodyweight gain in % of lean and DIO C57bl mice and GLP-1R-/- mice administered with VivoTag-750 liraglutide or vehicle BD for 4 consecutive days. Data represents means +/- SEM from 5 animals per group.

[P34]

For *in vivo* experiments, Vivotag750-liraglutide, Alexa594-liraglutide, and Alexa594-exenatide(9-39) were formulated as solutions in phosphate buffered saline for subcutaneous injection. Flourescently labeled Albagen™ (recombinant human albumin) (b) (4) The applicant synthesized [^{3}H-hexadecanoyl]liraglutide (batch 36J, specific activity 63 Ci/mmol, 89 mcg/mL, >98% radiochemical purity by HPLC). [^{125}I]GLP-1 (2200 Ci/mmol) was purchased from (b) (4)

Localization of Fluorescent Liraglutide in Mice

For acute treatment, male mice (wild-type (WT) C57bl or GLP-1R-/-, 5/strain/group) were administered a single subcutaneous dose of 120 nmol/kg Vivotag750-liraglutide (equivalent to 0.45 mg/kg liraglutide) and anesthetized with isoflurane 6 hours after dosing. For subchronic

dosing, male WT or GLP-1R-/- mice (5/strain/group) were subcutaneously administered 60 nmol/kg (equivalent to 0.23 mg/kg liraglutide) Vivotag750-liraglutide twice a day on the first day and 120 nmol/kg twice a day on days 2 to 4 or once a day with vehicle (phosphate buffered saline) for 4 days and anesthetized with isoflurane 6 hours after the last dose. Anesthetized mice were perfused with 10 mL heparinized saline followed by 10% neutral buffered formalin, then pancreas and brain were removed and immersed in 10% neutral buffered formalin at 4C overnight. For single plane illumination microscopy (SPIM), tissues were dehydrated with tetrahydrofuran, cleared with dibenzylether, sectioned, and Vivotag750 fluorescence was visualized using a 710 nm excitation source and monitoring emissions at 775 nm.

Figure 1 shows in pancreas of WT mice, the distribution of Vivotag750-liraglutide fluorescence was consistent with localization to islets while in GLP-1R-deficient GLP-1R-/- mice, weaker Vivotag750-liraglutide fluorescence was consistent with localization to blood vessels.

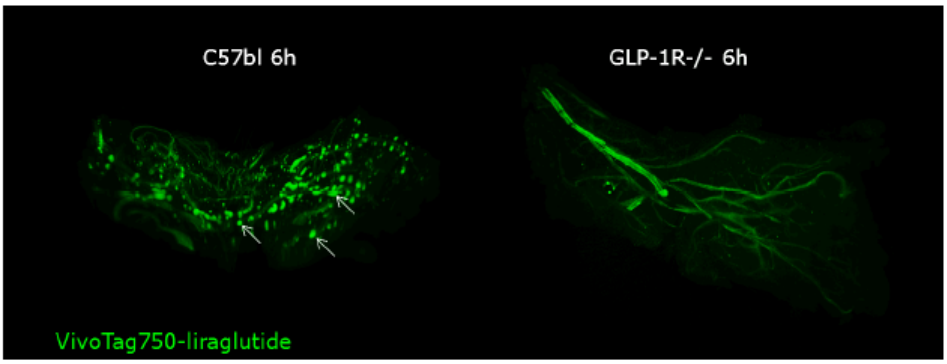

Figure 1 Vivotag750-liraglutide distribution in pancreas
Representative image of the fluorescent signal in pancreas from C57bl (n=5) and GLP-1R-/- (n=5) mice administered with VivoTag750-liraglutide. In C57bl mice, islets are clearly visible throughout the pancreas and a weaker signal can be observed in blood vessels. In GLP-1R-/- mice, the islet signal is absent. Arrows points to some, out of several, islets in the tissue.

[P17]

Reviewer note: The applicant should demonstrate the distribution of islets in pancreas is similar in WT and GLP-1R-/- mice.

Male WT or GLP-1R-/- mice were administered a single subcutaneous dose of 120 nmol/kg Alexa594-liraglutide (equivalent to 0.45 mg/kg liraglutide, 3/strain) or Alexa594-exendin(9-39) (2/strain). Six hours after dosing, mice were anesthetized with isoflurane, euthanized by transcardial perfusion with heparinized saline followed by perfusion with 10% neutral buffered formalin. Pancreata were removed, immersed in 10% neutral buffered formalin (overnight), transferred to 20% sucrose (to saturation), frozen in 2-methylbutane at -40C, and stored at -80C. Pancreata were cryosectioned (10 mm slices, 20C) and the sections were processed for fluorescence microscopy by staining with Hoechst nuclear stain and immunohistochemically staining for insulin using a guinea pig anti-insulin antibody and a secondary Cys2-labeled donkey anti-guinea pig antibody. Figure 2 shows Alexa594-liraglutide and Alexa594-exendin(9-39) (both fluoresce red) were detected in β cells (green fluorescent insulin(+) cells) in pancreata from WT C57bl mice (Figures 2B and 2D), but not in pancreata from GLP-1R-/- mice (Figures 2A and 2C). Higher magnification of pancreata from WT mice shows Alexa594-liraglutide was primarily localized to the cytoplasm (Figures 2E and 2E') while Alexa594-exendin(9-39) was primarily localized to the cell surface (Figures 2F and 2F').

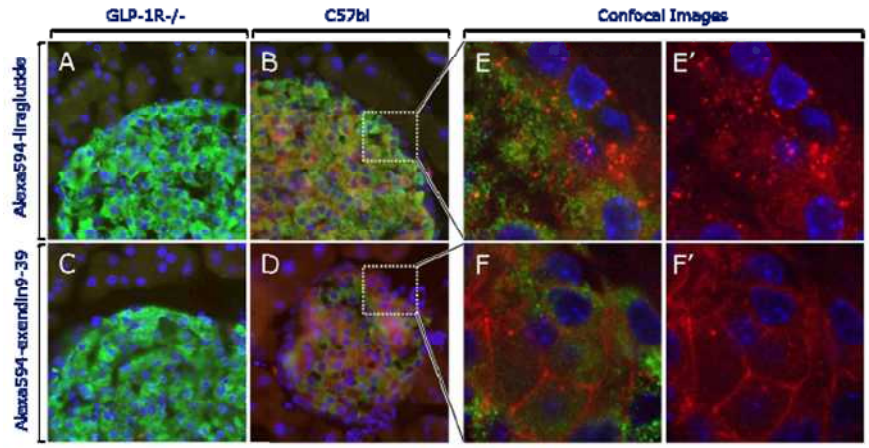

Figure 2 Alexa594-liraglutide and Alexa594-exendin(9-39) in pancreas
Representative images of mouse islets stained with Hoechst nuclear stain (blue), insulin (green) and Alexa594-liraglutide/Alexa594-exendin(9-39) (red). In mice lacking a functional GLP-1 receptor, no liraglutide or exendin(9-39) signal could be detected in insulin expressing beta-cells (A and C). In C57bl mice, both liraglutide and exendin(9-39) was detected in cells expressing insulin (B and D). High magnification confocal images revealed liraglutide in the insulin positive cytoplasm (E and E') while exendin(9-39) located to the plasma membrane (F and F').

[P17]

Reviewer note: Differences in subcellular localization of Alexa594-liraglutide and Alexa594-exendin(9-39) in pancreas cells from WT mice were consistent with GLP-1R agonist activity of liraglutide (resulting in receptor internalization) and antagonist activity of exendin(9-39).

Localization of Vivotag750-liraglute in brain was investigated in WT C57bl mice and GLP-1-/- mice. WT and GLP-1-/- mice were administered a single subcutaneous dose of 120 nmol/kg Vivotag750-liraglutide (equivalent to 0.45 mg/kg liraglutide) or treated for 4 days with escalating doses of up to 120 nmol/kg Vivotag750-liraglutide administered twice a day. As negative controls, WT mice were subcutaneously administered PBS once a day, and WT Balb/c mice were administered a single iv dose of 120 nmol/kg Vivotag750-albagen 4 hours prior to termination. Figure 3 shows autofluorescence in brain of mice administered a single dose of Vivotag750-liraglutide (Figure 3A), PBS (Figure 3C), or Vivotag750-albagen (Figure 3E) using 620/650 nm excitation/emission and localization Vivotag fluorescence at 710/750 nm excitation/emission in brain of mice administered Vivotag liraglutide (Figure 3B), Vivotag ablumin (Figures F), or PBS (Figures D). Vivotag750 fluorenscence was more extensive in mice administered Vivotag750-liraglutide (Figures 3B) compared to mice administered Vivotag750-albagen (Figure 3F).

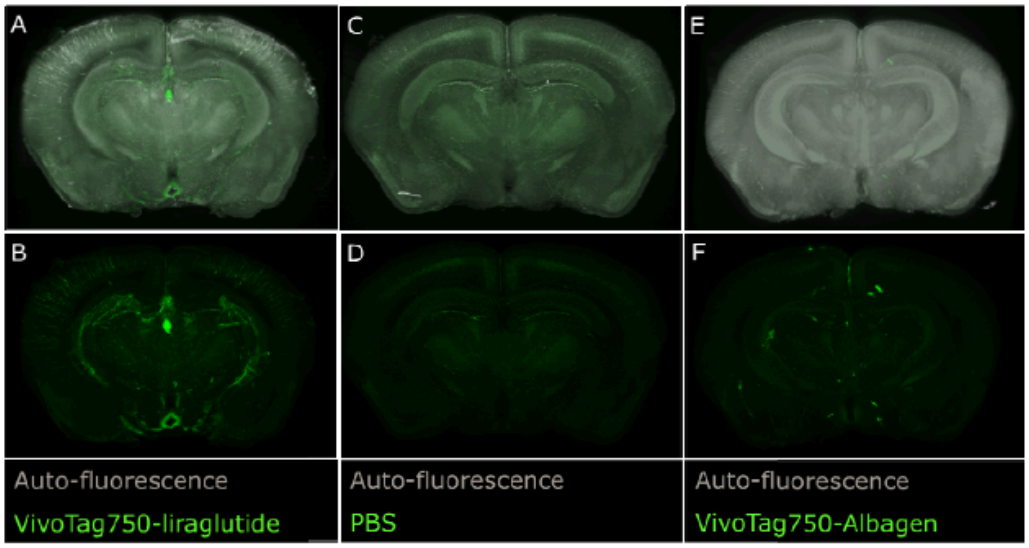

Figure 3 3D imaging of PBS and fluorescently labelled albagen in the brain
Representative image of a projection image of the fluorescent signal in the forebrain of mice administered with VivoTag750-liraglutide (A and B, n=5), PBS (C and D, n=5), or VivoTag750-albagen (E and F, n=3). Panel A, C, and E are sections scanned at 620nm and 710nm representing both auto-fluorescence from the tissue and specific signal. Panel B, D, and F are sections scanned at 710nm representing specific signal.

[P18]

Figures 4, 5, and 8 show after a single dose of Vivotag750-liraglutide, Vivotag750 fluorescence occurred in circumventricular organs (area postrema, choroid plexus, subfornical organ, median eminence, and organum vasculosum of the lamina terminalis) in WT mice, but not in GLP-1R-/- mice. Figure 4 shows Vivotag750-liraglutide-related flourescence occurred in the area postrema of brain from 5/5 WT C57bl mice (Figures 4B and 4B'), but not in brain from GLP-1R-/- mice (Figures 4D and 4D'). GLP-1R-independent Vivotag750-liraglutide fluorescence occurred in other regions of the brainstem with a similar distribution in both WT and GLP-1R-/- mice (Figures 4B' and 4D', respectively). Figure 8 shows quantitaitve assessment of fluorescence intensity in the area postrema in brain from WT mice administered a single dose of Vivotag750-liraglutide and in brain from WT and GLP-1R-/- mice adminsitered Vivotag750 liralgutide for 4 days. In the area postrema in brain from mice adminsitered Vivotag750-liraglutide, geometric mean fluorescence intensity was similar in WT mice 6 hours after a single dose or 6 hours after the last dose during 4 days of treatment, but geometric mean fluorescence was 90- to 112-fold higher from WT mice (single dose or 4 days of treatment with Vivotag750-liraglutide) compared to GLP-1R-/- mice (4 days of treatment with Vivotag750-liraglutide).

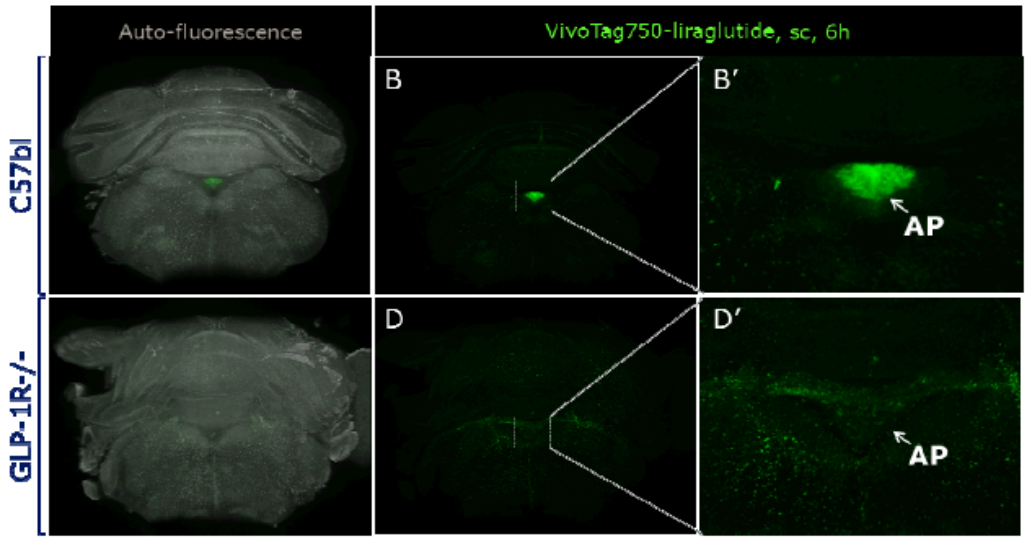

Figure 4 Vivotag750-liraglutide distribution in the brainstem
3D Projection images of the fluorescent signal in the brain stem of C57bl (n=5) (A, B and B') or GLP-1R-/- (n=5) (C, D and D') mice administered with VivoTag750-liraglutide. Sections A and C are scanned at 620nm and show general auto-fluorescence. Sections B and D are scanned at 710nm and show the 750nm specific signal. Panel B' and D' are enlargements of the AP in B and D respectively. Abbreviations: AP: Area Postrema

[P19]

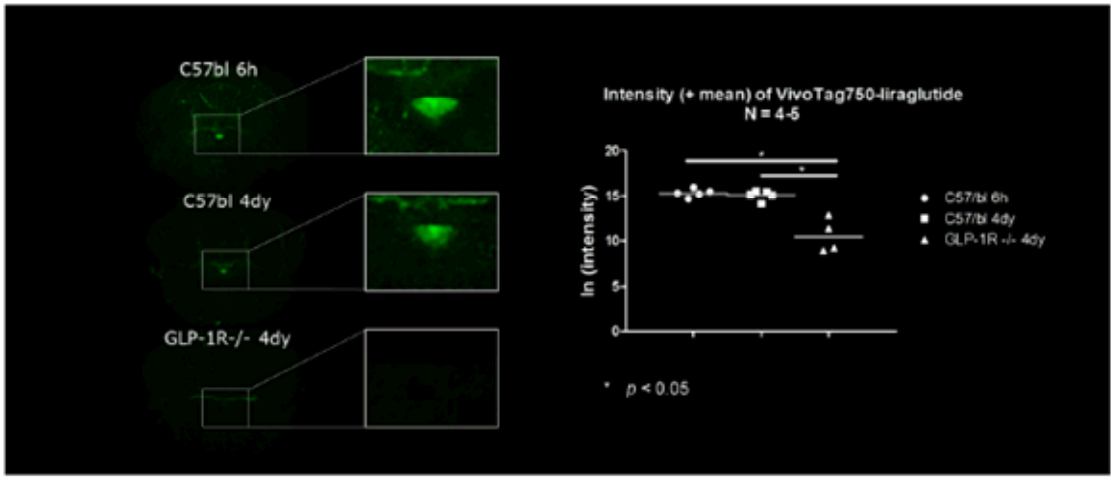

Figure 8 Quantification of Vivotag750-liraglutide in AP
VivoTag750-liraglutide signal in AP in the brainstem with means (grey horizontal lines). The inserts are enlargements of the projection image representing AP in C57bl or GLP-1R-/- mice treated acutely or chronically with VivoTag750-liraglutide. The fluorescent intensity in AP is quantified in the scatter plot to the right as ln intensities.

[P22-23]

Figure 5 shows after a single dose of Vivotage750-liraglutide in WT and GLP-1R-/- mice, Vivotag750-liraglutide-related fluorescence was observed in the zona interna (ZI) of the median eminence (ME), subfornical organ (SFO), arcuate nucleus (Arc) and the paraventricular nucleus (PVN) in the forebrain of WT mice (Figures 5B, 5B', and 5B''), but not in the same regions in

GLP-1R-/- mice (Figure 5D, 5D', and 5D'') while Vivotage750-liraglutide fluorescence intensity in the choroid plexus (ChP) was similar in WT and GLP-1R-/- mice (Figures 5B and 5D).

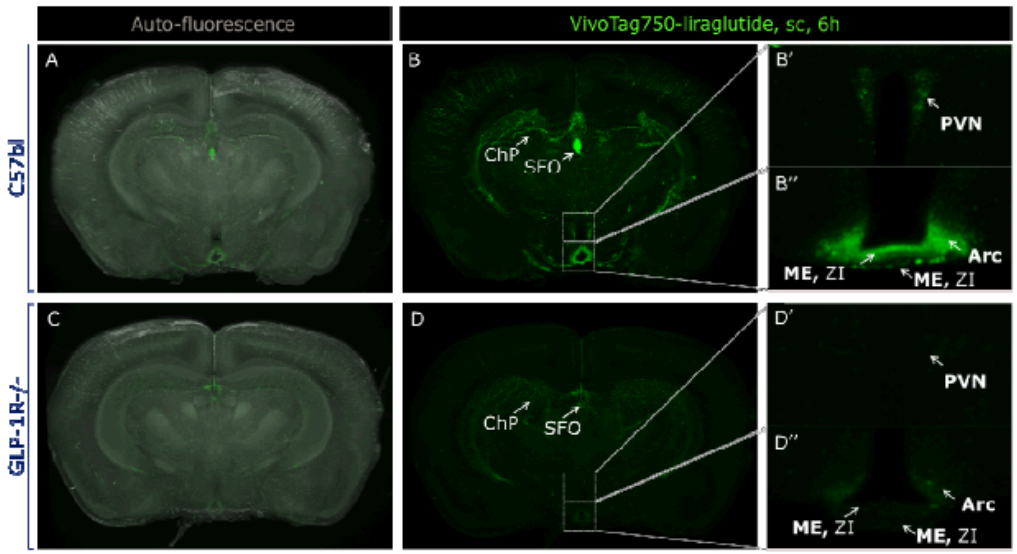

Figure 5 Vivotag750-liraglutide distribution in the forebrain

Representative image of a fluorescent signal in forebrain of C57bl (A, B, B', B'') or GLP-1R-/- (C, D, D', D'') mice administered with VivoTag750-liraglutide. Panels B', B'', D' and D'' are enlargements of a single section with PVN or ME/Arc. Sections are scanned at 620nm and 710nm representing both auto-fluorescence from the tissue (A and C) and specific signal (B, B', B'', D, D' and D''). Abbreviations: Arc: Arcuate Nucleus, ChP: Choroid Plexus, ME: Median Eminence, PVN: Paraventricular hypothalamic Nucleus, SFO: Subfornical Organ, ZE: Zona Externa, ZI: Zona Interna

[P20]

Figure 6 shows Vivotag750-liraglutide-related fluorescence characterized as small thread-like structures occurred in the zona interna and zona externa of the median eminence in both WT and GLP-1R-/- mice (Figures 6A and 6B, respectively). Fluorescence intensity in the arcuate nucleus (Arc, Figure 9), median eminence (ME, Figure 9), and paraventricular nucleus (PVN, Figure 10) was quantitatively assessed in brain from WT mice administered a single dose of Vivotag750-liraglutide and in brain from WT and GLP-1R-/- mice adminsitered Vivotag750 liralgutide for 4 days. In the arcuate nucleus in brain from mice adminsitered Vivotag750-liraglutide, geometric mean fluorescence intensity was similar in WT mice 6 hours after a single dose or 6 hours after the last of dose during 4 days of treatment, but geometric mean fluorescence intensity was 9.2-fold higher from WT mice (4 days of treatment with Vivotag750-liraglutide) compared to GLP-1R-/- mice (4 days of treatment with Vivotag750-liraglutide). In the median eminence from mice adminsitered Vivotag750-liraglutide, geometric mean fluorescence intensity was similar in WT mice 6 hours after a single dose or 6 hours after the last dose during 4 days of treatment, but geometric mean fluorescence intensity was 11.7-fold higher from WT mice (4 days of treatment with Vivotag750-liraglutide) compared to GLP-1R-/- mice (4 days of treatment with Vivotag750-liraglutide). In the paraventricular nucleus in brain from mice adminsitered Vivotag750-liraglutide, geometric mean fluorescence intensity was 3.1-fold lower in WT mice 6 hours after a single dose compared to 6 hours after the last of dose during 4 days of treatment, and geometric mean fluorescence intensity was 225-fold higher from WT mice (4 days of treatment with Vivotag750-liraglutide) compared to GLP-1R-/- mice (4 days of treatment with Vivotag750-liraglutide).

Reviewer note: In WT C57bl mice, a ~3-fold increase in fluorescence intensity in the paraventricular nucleus in the hypothalmus after 4 days of treatment with Vivotag750-liraglutide compared to fluorescence intensity after a single dose suggests repeat dosing with liraglutide upregulates GLP-1R in the PVN.

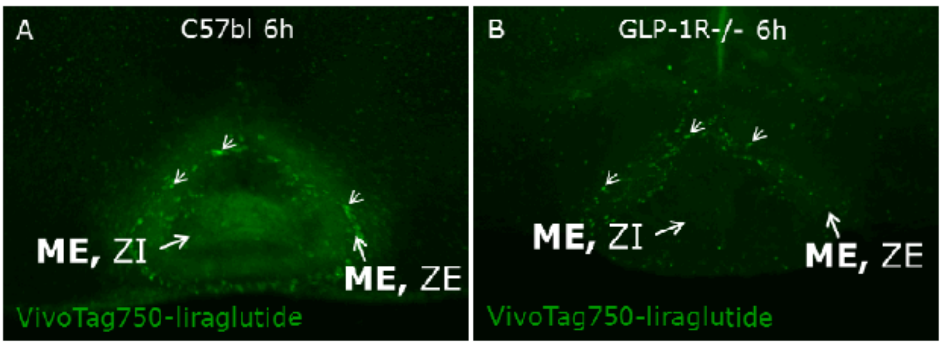

Figure 6 Vivotag750-liraglutide distribution in Arc and ME
Representative image of a projection image of the fluorescent signal in Arc and ME from C57bl (A) and GLP-1R-/- (B) mice dosed with VivoTag750-liraglutide. A signal is observed in ZI of ME in C57bl (A). In GLP-1R-/- mice, the signal is restricted to small thread-like structures (small, white arrows in B) in ZE of ME which are also visible in C57bl mice (small, white arrows in A). Sections are scanned at 710nm. Abbreviations: ME: Median Eminence, ZE: Zona Externa, ZI: Zona Interna
[P21]

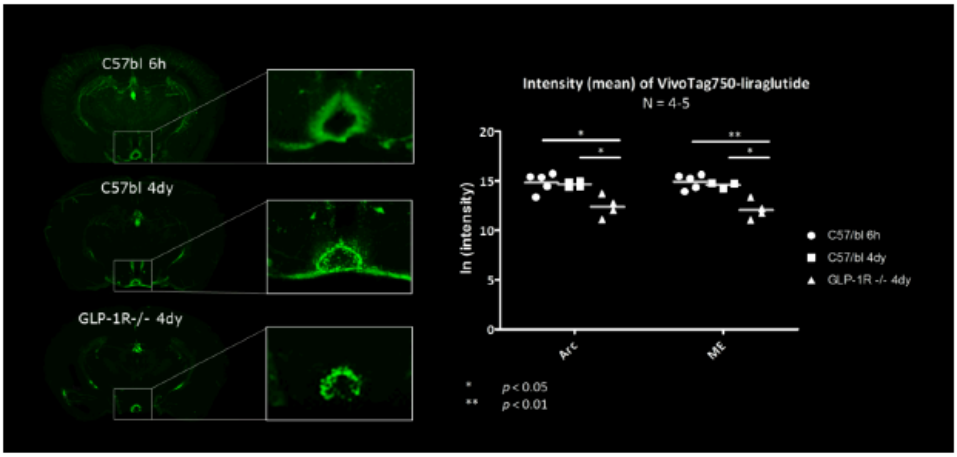

Figure 9 Quantification of Vivotag750-liraglutide in Arc and ME
VivoTag750-liraglutide signal in ME and Arc in the hypothalamus with geometric means (grey horizontal lines). The inserts are enlargements of the projection image representing the ME/Arc region in C57bl or GLP-1R-/- mice treated acutely or chronically with VivoTag750-liraglutide. The fluorescent intensity in the ME/Arc region is quantified in the scatter plot to the right as ln intensities.
[P23]

Figure 10 also shows a small area of fluorescence ventral to the paraventricular nucleus, probably a larger structure of the hypothalamic subnuclei, occurred in WT C57bl mice (single dose or 4 days of treatment with Vivotag750-liraglutide), but not in GLP-1R-/- mice (4 days of treatment with liraglutide).

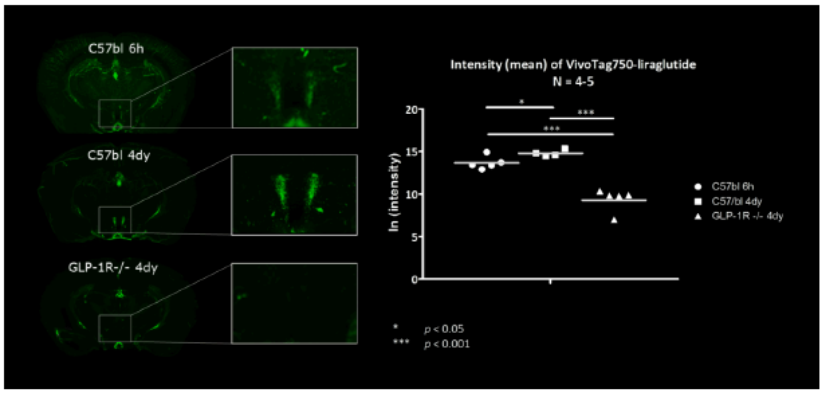

Figure 10 Quantification of Vivotag750-liraglutide in PVN
VivoTag750-liraglutide signal in PVN in the hypothalamus with geometric means (grey horizontal lines). The inserts are enlargements of the projection image representing PVN in C57bl or GLP-1R-/- mice treated acutely or chronically with VivoTag750-liraglutide. The fluorescent intensity in PVN is quantified in the scatter plot to the right as ln intensities.

[P24]

Diffuse Vivotag750-liraglutide-related fluorescence in the dorsomedial hypothalmic nucleus (DMH, Figure 11) and fluorescence in the supraoptic nucleus (Figure 12) occurred in 5/5 WT mice administered Vivotag750-liraglutide for 4 days.

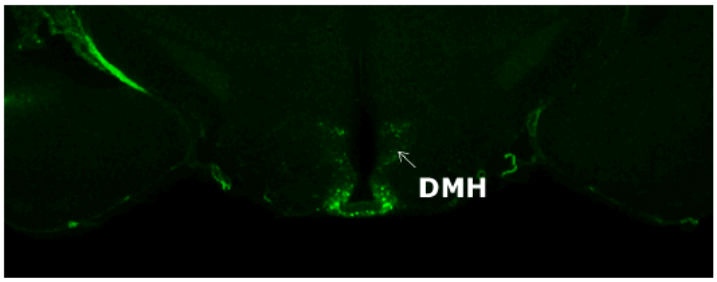

Figure 11 Vivotag750-liraglutide distribution in dorsomedial hypothalamic nucleus
Representative image of an enlargement of a single section representing the dorsomedial hypothalamic nucleus (DMH) in C57bl mice treated chronically with VivoTag750-liraglutide. The single section originates from a series of stacks scanned at 710nm.

[P25]

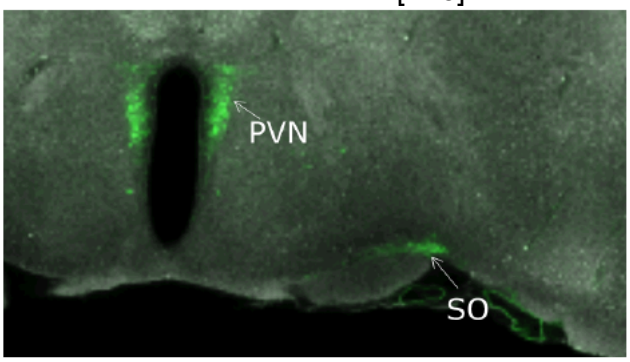

Figure 12 Vivotag750-liraglutide distribution in supraoptic nucleus
Representative image of an enlargement of a single section representing the supraoptic nucleus (SO) in C57bl mice treated chronically with VivoTag750-liraglutide. The single section originates from a series of stacks scanned at 620 and 710nm.

[P25]

In the frontal lobe, Vivotag750-liraglutide-related fluorescence occurred in the organum vasculosum of lamina terminalis (OVLT) in all WT mice (5/5), but only a weak fluorscence signal was observed in 2/5 GLP-1R-/- mice (Figure 7B' and 7D').

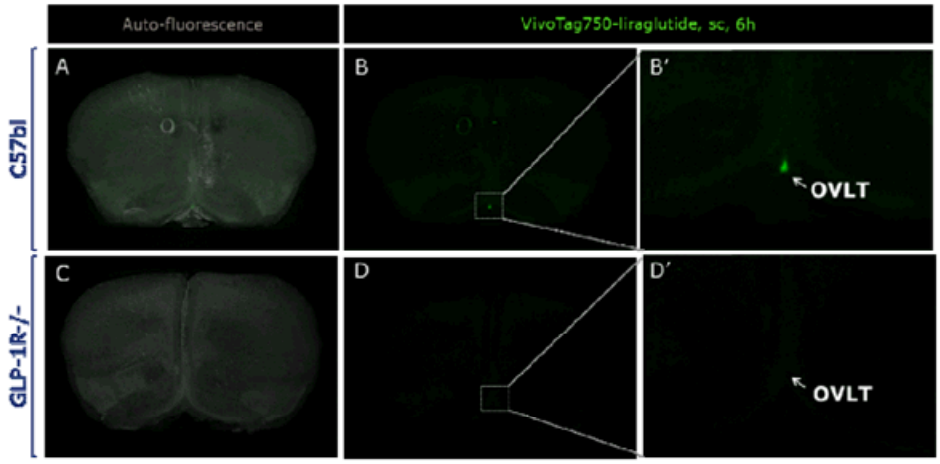

Figure 7 Vivotag750-liraglutide distribution in the frontal lobe

Representative projection images of the fluorescent signal in the frontal lobe of C57bl (A, B and B') or GLP-1R-/- (C, D and D') mice dosed with VivoTag750-liraglutide. Panels B' and D' are enlargements of single sections representing OVLT. Sections are scanned at 620nm (A and C) and 710nm (B, D, B' and D') representing both auto-fluorescence from the tissue and the specific signal. Abbreviations: OVLT: Organum Vasculosum of Lamina Terminalis.

[P22]

Areas of the brain specifically stained with Vivotag750-liraglutide in WT C57bl mice were the paraventricular nucleaus, arcurate nucleaus, doromedial hypothalamic region, supraoptic nucleus, median emminence zona interna, subfornical organ, and organum vasculosum of lamina terminalis in the forebrain and area postrema in the brainstem. Areas of the brain stained with Vivotag750-liraglutide in WT C57bl mice and GLP-1R-/- mice were the median emminence zona externa and choroid plexus.

Table 1 Vivotag750-liraglutide signal in the brain

Presence (+) or absence (-) of fluorescent signal in the mouse brain following peripheral injection with VivoTag750-liraglutide. Black indicates if the signal is only seen in mice with functional GLP-1R expression.

Region	Brain area		Signal in C57bl	Signal in GLP-1R-/-
Forebrain	PVN		+	-
	Arc		+	-
	DMH		+	-
	SO		+	-
	ME	ZE	+	+
		ZI	+	-
	SFO		+	-
	ChP		+	+
	OVLT		+	-
Brain stem	AP		+	-

[P26]

Colocalization of neurons expressing cocaine and amphetamine regulated peptide (CART) and the GLP-1R was evaluated in brains from rats intravenously administered a single dose of 120 nmol/kg Alexa594-liraglutide (red fluorescence, Figure 13) that were stained using Hoechst nuclear stain (blue, Figure 13) and and anti-CART antibody (green, Figure 13B, C, C', D, and D')) or a nonspecific IgG (green, Figure 13A). For this study, the rat was selected because CART neurons are better characterized in rats compared to mice. The majority of CART(+) cells in the arcuate nucleus (Figures 13B, C, and D) were also labeled with Alexa594-liraglutide (Figure 13C' and 13D') indicating CART neurons express the GLP-1R.

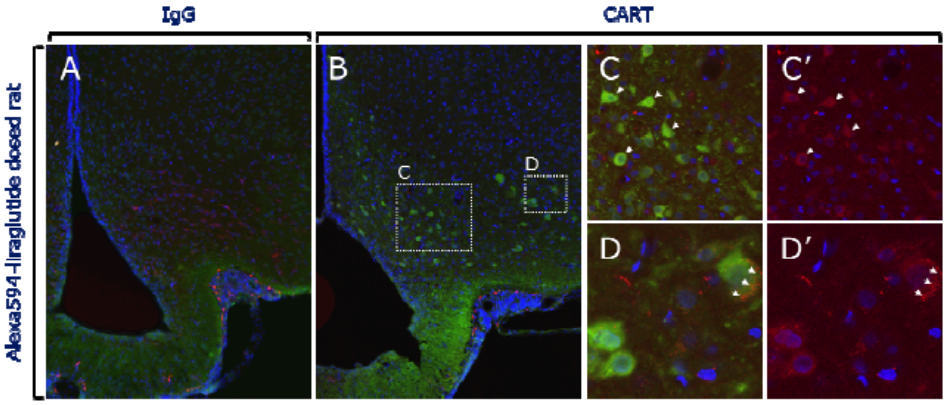

Figure 13 Co-localisation of Alexa594-liraglutide with CART

Rat hypothalamus stained with Hoechst nuclear stain (blue), CART (green) and Alexa594-liraglutide (red) (B, C, C', D, D') or with IgG (green) (A). C, C', D and D' shows high magnification confocal images of Alexa594-liraglutide accumulation in the CART cells. C and D includes all channels (red, blue, green) whereas C' and D' are the same images as C and D omitting the green (CART specific) channel. Arrows point to some of the CART/Alexa594-liraglutide positive cells (C, C') and to Alexa594-liraglutide in the cytoplasm of a CART positive cell (D, D').

[P27]

Reviewer note: Characterization of the rabbit anti-CART polyclonal antibody (Ca7-OVA, (b) (4)) (b) (4) was not reported.

Localization of Radiolabeled Liraglutide in Mouse and Rat Brain

Brain regions expressing GLP-1Rs were evaluated by micro-autoradiography of brain slices from NMRI mice (4/dose) administered a single iv injection of 0.2 or 0.4 mg/kg [^{3}H-hexadecanoyl]liraglutide (23.7 mCi/mg liraglutide, in PBS) or Sprague Dawley rats (n = 8) administered a single subcutaneous dose of 0.15 mg/kg [^{3}H-hexadecanoyl]liraglutide (23.7 mCi/mg liraglutide, in PBS). Isoflurane-anesthetized mice were euthanized by transcardial perfusion with saline 2 hours after dosing and isoflurane-anesthetized rats were euthanized by transcardial perfusion with saline 8 hours after dosing. After transcardial perfusion, brains from mice and rats were dissected, frozen in 2-methyl butane at -40C, and stored at -80C until processing for autoradiography. [^{3}H-hexadecanoyl]Liraglutide-related radioactivity was observed in the arcuate nucleus, median eminence, and area postrema in mice (Figure 14) and median eminence, arcuate nucleus, and paraventricular nucleus in rats (Figure 15).

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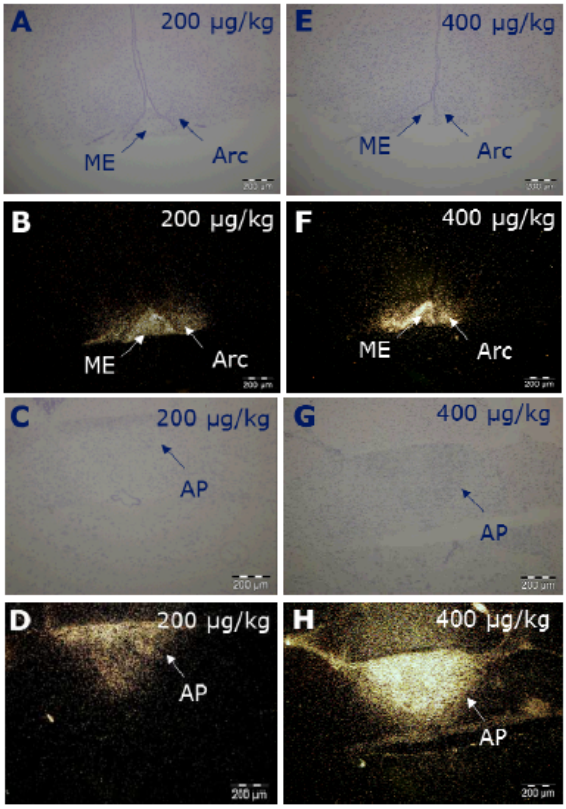


Figure 14 ^{3}H-liraglutide distribution in the mouse brain

Representative images of ^{3}H-liraglutide brain distribution in mouse following peripheral injection. Enlargement of hypothalamic and brainstem regions. Pictures A, C, E and G are light field micrographs of brain sections from mice dosed with two different doses of ^{3}H liraglutide. Pictures A, B, C, D are sections from mice dosed with 200 µg/kg, pictures E, F, G, H are sections from mice dosed with 400 µg/kg. Pictures B, D, F and H show dark field images of A, C, E and G, respectively. Abbreviations: AP: Area postrema, Arc: Arcuate nucleus, ME: Median eminence.

[P28]

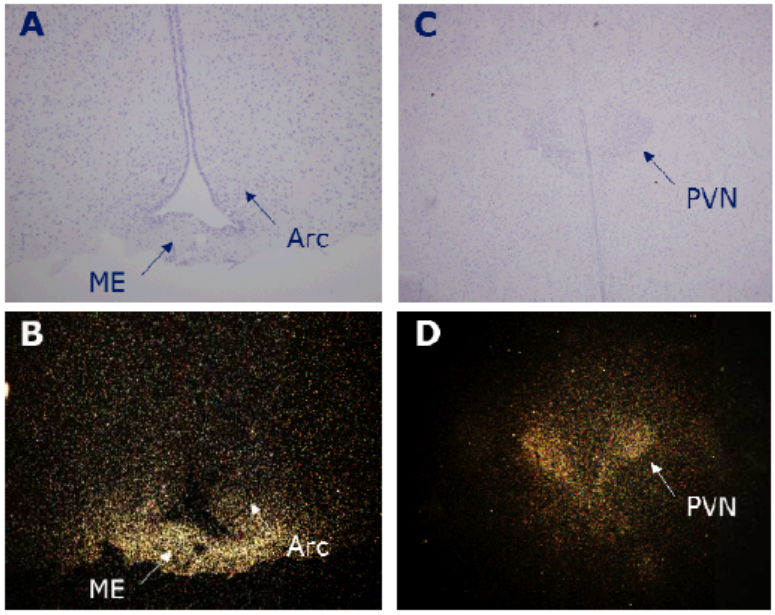


Figure 15 ^{3}H-liraglutide distribution in the rat brain

Representative images of ^{3}H-liraglutide brain distribution in rat following peripheral injection. The figure shows enlargements of the hypothalamic region. Pictures A and C are light field micrographs of brain sections from rats dosed with ^{3}H-liraglutide. Pictures B and D show dark field images of A and C. Abbreviations: Arc: Arcuate nucleus, ME: Median eminence, PVN: Paraventricular hypothalamic nucleus.

[P29]

Evalution of Blood Brain Barrier Penetration by Capillary Depletion of Brain from Radiolabeled Liraglutide-treated Mice

For capillary depletion studies assessing blood brain barrier penetration of liraglutide, NMRI mice were adminsitered a single iv dose of 0.20 or 0.40 mg/kg [^{3}H-hexadecanoyl]liraglutide (23.7 mCi/mg liraglutide, in PBS), anesthetized wtith isoflurane, and euthanized by transcardiac perfusion with saline 2 hours after dosing. Dissected brains were stored in ice-cold capillary depletion buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 10 mM glucose, pH 7.4). Brains were homogenized in PBS, dextran was added to a final concentration of ~12%, and brains were homogenized a second time to produce whole brain homogenate (brain + capillaries). Capillaries were removed from a aliquot of the whole brain homogenate by centrifugation at 5400 x g for 15 minutes at 4C. [^{3}H]Liraglutide-related radioactivity was quantified by liquid scintillation spectroscopy of whole brain homogenate and parenchyma (capillary-free brain homogenate). Figure 16 (below) shows radioactivity in whole brain and parenchyma increased with [^{3}H]liraglutide dose. Radioactivity in whole brain was 1.76-fold higher than parenchyma after dosing with 0.2 mg/kg [^{3}H]liraglutide and 1.44-fold higher after dosing with 0.4 mg/kg [^{3}H]liraglutide indicating a significant fraction of liraglutide-related radioactivity was associated with capillaries.

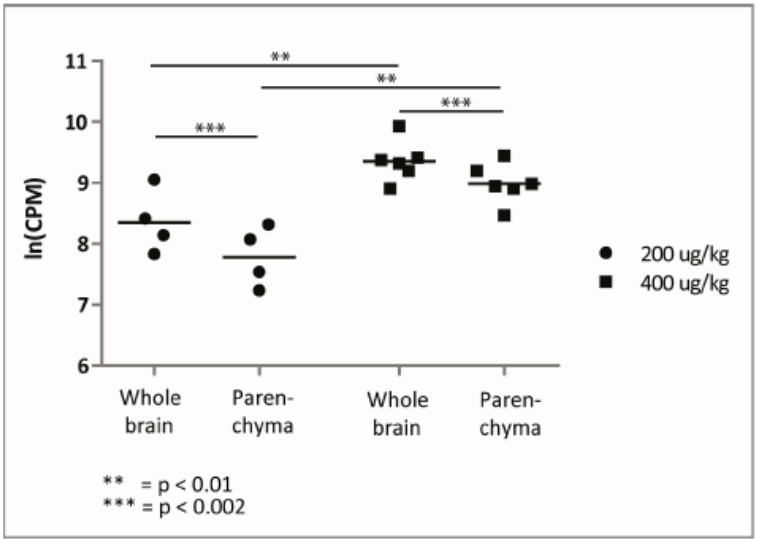


Figure 16 Detection of ^{3}H-liraglutide in brain from mice by capillary depletion. Individual measurement of radioactivity in whole brain (before separation from capillaries) and parenchyma (brain fraction following separation from capillaries) respectively from mice administered with two different doses of ^{3}H-liraglutide.

[P30]

Study Title: Mapping of GLP-1 receptor expression in the non-human primate brain (report GUS2012-059-NN)

Key Study Findings

- The GLP-1R in brain of male rhesus monkeys was mainly distributed along a functional continuum of areas involved in regulation of appetite and body weight including the area postrema, nucleus of the solitary tract, lateral parabrachial nucleus, amygdala, bed nucleus of the stria terminalis, and the paraventricular nucleus, dorsomedial nucleus, and arcuate nucleus of the hypothalamus. In the brains of monkeys, GLP-1R transcript (determined by in situ hybridization), protein (determined by immunohistochemical staining), and ligand binding activity (determined by in situ radioligand binding) generally colocalized with high levels of GLP-1R expression in the brainstem and hypothalamus.
  - The highest levels of GLP-1R transcript, protein, and ligand binding activity in the brain stem occurred in the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus. GLP-1R was also expressed in the lateral parts of the reticular formation, lateral parabrachial nucleus, the dorsal raphe, A1/C1, and the gigantocellular nucleus.
  - The highest levels of GLP-1R transcript, protein, and ligand binding activity in the hypothalamus occurred in cell bodies and fibers of the paraventricular, dorsomedial, and arcuate nuclei.
- High levels of GLP-1R protein occurred in the central nucleus of the amygdala and the bed nucleus of the stria terminalis with lower levels in the medial nucleus of the amygdala and the nucleus accumbens.
- Low levels of GLP-1R transcript, protein, and binding activity occurred in the paraventricular nucleus of the thalamus
- GLP-1R was not detected in the cerebral cortex (frontal, occipital, temporal, or parietal).
- Study deficiencies were:
  - Specificity of [^{125}I]GLP-1 binding in tissue sections was not demonstrated.

- Although the applicant showed areas of the caudal brainstem binding [^{125}I]GLP-1 and areas stained by the mouse anti-GLP-1R monoclonal antibody were very similar, the extensive characterization of the anti-GLP-1R monoclonal antibody (3F52) was not included in this report, although a study demonstrating GLP-1Rspecificity of 3F52 was recently published (Pyke et al, Endocrinology. 2014 Apr;155(4):1280-90).

Summary

GLP-1 receptor (GLP-1R) distribution in brains of monkeys was evaluated by immunohistochemical (IHC) staining with an anti-GLP-1R monoclonal antibody, by in situ hybridization (ISH) of GLP-1R mRNA with RNA probes, and by in situ GLP-1 radioligand binding (ISLB) using perfusion fixed brain from male rhesus macaques obtained from (b) (4) .

Paraformaldehyde-fixed brains (fixed during euthanasia by perfusion with saline followed by 4% buffered paraformaldehyde, pH 7.4) were blocked into sections (hypothalamus / thalamus, prefrontal cortex, temporal cortex containing hippocampus, parietal cortex, occipital cortex, cerebellum, midbrain, and hindbrain) and the blocks were post-fixed in 4% paraformaldehyde saturated with 25% sucrose for freezing at -80C prior to sectioning. Brain areas were sliced into 25 μm thick sections with neighboring sections used for ISH, ISLB, and IHC staining. Tissue sections were counterstained with thionin for light microscopy.

For ISH, radioactive RNA probes were synthesized with a human GLP-1R cDNA template using RNA polymerase to incorporate [^{33}P]UTP. Tissue sections were fixed in 4% paraformaldehyde and acetylated in triethanolamine and acetic anhydride prior to dehydration in an ethanol gradient. Tissue sections were hybridized with the denatured [^{33}P]RNA probe, exposed to RNAse to remove single stranded RNA, and brain tissue sections were exposed to autoradiographic film in the absence of emulsion (9 day exposure) or in the presence of emulsion (21 day exposure). Developed films from emulsion-treated and emulsion-free exposures were digitized using a high resolution scanner while emulsion-treated slides were microscopically examined.

For ISLB performed on tissue sections neighboring those used for ISH, tissue sections were preincubated in binding buffer followed by incubation with 0.3 nM [^{125}I]GLP-1 in the absence and presence of 100 nM unlabeled GLP-1 to determine total and nonspecific radioligand binding, respectively. After several washes and short exposure in 80% ethanol to dehydrate samples, dried tissue sections were exposed to film (days), the films were developed, dried, and digitized using a high resolution scanner. Tissue sections were then dipped into K5 emulsion diluted in water, dried, exposed for 19 days, developed (D19 developer from Kodak), development was halted (1% acetic acid, 1% glycerol) and fixed in 30% sodium thiosulfate. Tissue sections were dehydrated in 70% ethanol, counterstained with Cresylviolet dehydrated in up to 96% ethanol, and mounted in Pertex for digital imaging using a Hamamatsu NanoZoomer 2.0HT at 40X magnification.

For IHC staining using fluorescyl-tyramide signal amplification, neighboring tissue sections were fixed in 4% paraformaldehyde, rinsed, treated with 1% H_2O_2, incubated with a primary mouse anti-GLP-1R monoclonal antibody (3F52) or a negative control mouse monoclonal antibody (LAGH, antibody specificity unknown), washed, labeled with an anti-mouse-HRP (b) (4)), and treated with CSA II, a biotin-free fluorescyl-tyramide signal amplification system ((b) (4)), prior to counterstaining with Hoechst nuclear stain and mounting with (b) (4) FMM. Digital images of stained tissue sections were obtained using a Hamamatsu NanoZoomer 2.0HT at 40X magnification. For IHC staining using biotinylated tyramide amplification and DAB / Ni chromagen, tissue sections were treated with 1% H_2O_2, Avidin Block ((b) (4) then Biotin Block ((b) (4) and rinsed prior to incubating with the primary mouse anti-GLP-1 monoclonal antibody 3F52. To visualize GLP-1R immunoreactivity, 3F52-stained tissue sections were rinsed, incubated with a secondary

biotinylated donkey anti-mouse antibody ([redacted]), washed, incubated in A/B solution ([redacted] washed, incubated with biotinylated tyramide for signal amplification, rinsed, incubated in A/B solution, and rinsed prior to incubation with DAB / Ni solution [redacted]), washed, mounted on gel-subbed slides, dried, dehydrated in ethanol and xylene, and mounted with Permount. Digital images of stained tissue sections were obtained using an Olympus Slide scanner at 10X magnification (Figures 2, 3, 4, 7, 10, 12, and 13).

To validate the IHC staining method, immunostaining observed by fluorescence microscopy of caudal brain stem tissue sections containing the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus (DMX) stained using the fluorescyl-tyramide signal amplification IHC staining method using the primary mouse anti-GLP-1R monoclonal antibody (clone 3F52) were compared to the distribution of [^{125}I]GLP-1 binding sites determined by ISLB using similar tissue sections. Areas stained by the anti-GLP-1R antibody overlapped with [^{125}I]GLP-1 binding areas (Figure 1, below). [^{125}I]GLP binding overlaps with areas of high signal intensity detected by GLP-1R IHC staining in the DMX, NTS and AP.

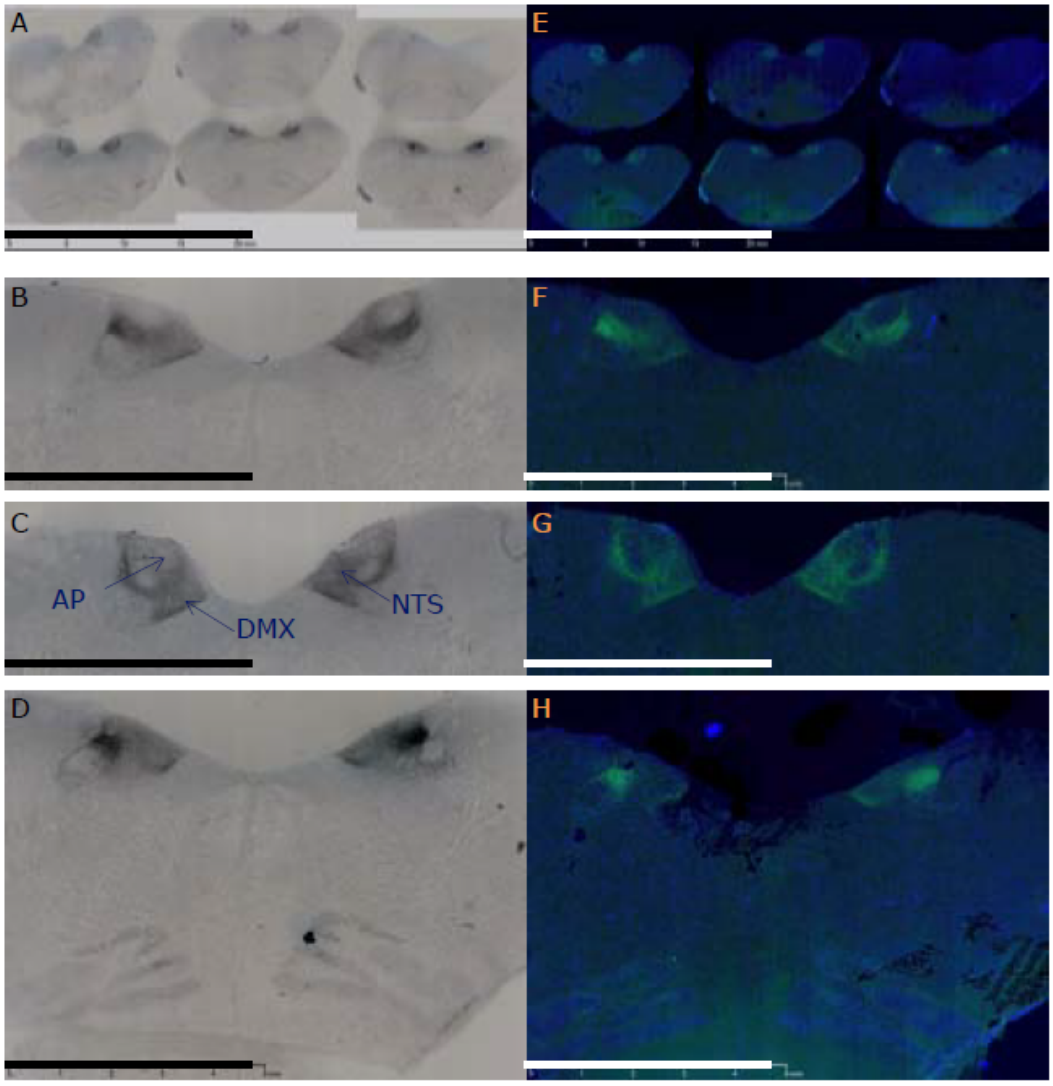


Figure 1 GLP-1R expression in the brainstem

In situ ligand binding (ISLB, left column) and immunohistochemistry (IHC, right column) from the brainstem at the level of the dorsal vagal complex demonstrating complete overlap between ISLB using ^{125}I-GLP-1 and IHC using the monoclonal antibody 3F52 raised against the extracellular domain of the human GLP-1R. Intense labelling is seen in the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX) and the area postrema (AP). Scale bars: A and E=20mm; B, C, D F, G and H=5mm)

[P15]

Semi-quantitative GLP-1R transcript levels (determined by ISH), protein levels (determined by IHC staining), and levels of functional receptor (determined by ISLB) in regions of brain from male rhesus monkeys are summarized in Table 1 and compared to levels in brain regions of rats reported in published studies (Merchenthaler et al, J Comp Neurol. 403:261-280, 1999; Goke et al, Eur J Neurosci. 7:2294-2300, 1995; Goke et al, Neuroendocrinology. 62:130-134, 1995; Orskov et al, Diabetes. 45:832-835, 1996).

GLP-1R was not detected in the monkey cerebral cortex (parietal, temporal, frontal, or occipital) by IHC staining, ISH, or ISLB.

In the amygdala, GLP-1R transcript, protein, and ligand binding activity was detected in the central nuclei and medial nuclei and bed nucleus of the stria terminalis. GLP-1R protein was

detected in both cell bodies and fibers (presumably representing both dendrites and terminal fields) of the medial and central nuclei by IHC staining.

In the striatum and basal ganglia, GLP-1R expression was detected in the nucleus accumbens (by IHC staining of cell bodies (with long dendrites) and fibers, ISH, and ISLB) and in the striatum internal globus pallidus (by IHC staining of cell bodies and fibers, ISH, and ISLB). A strong ISLB signal was detected in the islands of Calleja, but the presence of GLP-1Rs in this area was not confirmed by ISH or IHC staining. GLP-1R expression was detected in bed nucleus of the stria terminalis (by IHC staining of cell bodies and fibers, ISH, and ISLB).

In the septum, GLP-1 expression was detected in the lateral septum and medial septum (by IHC staining of cell bodies and fibers, ISH, and ISLB).

In the diencephalon (hypothalamus and thalamus), GLP-1R expression was detected in the paraventricular nucleus of the thalamus (by IHC staining of cell bodies and fibers, ISH, and ISLB) and in the paraventricular nucleus, supraoptic nucleus (label primarily occurred on fibers), medial preoptic area, lateral hypothalamic area, dorsomedial nucleus, arcuate nucleus, and median eminence of the hypothalamus (by IHC staining of cell bodies and fibers, ISH, and ISLB). In the hypothalamus, the highest GLP-1R levels detected by ISLB, ISH, and IHC staining occurred in the paraventricular, supraoptic, and arcuate nuclei.

In the mesencephalon, GLP-1R was detected in the ventral tegmental area, the substantia nigra, the posterodorsal tegmental nucleus, periaqueductal grey, dorsal raphe, and substantia nigra (by IHC staining of cell bodies and fibers, ISH, and ISLB) with the highest levels of GLP-1R mRNA, protein, and ligand binding activity occurring in the posterodorsal tegmental nucleus.

In the pons, GLP-1R was detected in the lateral reticular formation, the parabrachial nucleus, and locus coeruleus (by IHC staining of cell bodies and fibers, ISH, and ISLB) with similar levels of [^{125}I]GLP-1 binding activity in all these regions, but higher levels of GLP-1R antibody staining occurred in fibers in the lateral reticular formation and parabrachial nucleus.

In the medulla, GLP-1R transcript (detected by ISH), protein in fibers (detected by IHC staining), and ligand binding activity (detected by ISLB) occurred in the nucleus of the solitary tract (NTS), area postrema (AP), dorsal motor nucleus of the vagus, A1/C1 area, gigantocellular nucleus, and inferior olive. GLP-1R protein was detected in cells (by IHC staining) of the NTS, AP, DMX, and gigantocellular nucleus, but not in the cell bodies of the A1/C1 or the inferior olive. In the medulla, the highest levels of ligand binding occurred in the NTS, AP and DMX with the highest levels of GLP-1R protein detected by IHC staining occurring in fibers of the NTS and AP and the highest levels of transcript occurring in the AP.

In the spinal cord, GLP-1R binding activity (by ISLB) and receptor protein (by IHC staining) were detected in fibers of the dorsal horn, but the receptor transcript was not detected.

In the cerebellum, GLP-1R transcript, protein, and ligand binding activity were detected in the medial cerebellar nuclei.

In the brain of male rhesus monkeys, the distribution of GLP-1R transcript, protein, and ligand binding activity generally colocalized with high levels in hypothalamic nuclei including the paraventricular nucleus, arcuate nucleus, dorsomedial hypothalamic nucleus, and in the lateral hypothalamic area. GLP-1R localization in the hypothalamus and caudal brainstem in monkey was consistent with a role for GLP-1 in regulating food intake and body weight. The distribution of GLP-1R in the brain of male rhesus monkeys was similar to the distribution in the rat brain. In the monkey paraventricular nucleus, high levels of GLP-1R immunoreactivity mainly occurred in fiber-like structures, probably representing terminals or dendrites, similar to findings in the rat. The arcuate nucleus in the forebrain contains the most dense GLP-1 innervation in the monkey with high levels of GLP-1R immunoreactivity occurring in a dense plexus of fibers and cell bodies, probably on α-MSH/CART neurons, which may mediate the anorexigenic effects of centrally administered GLP-1.The dorsomedial hypothalamic nucleus, which projects to the paraventricular nucleus, also expressed GLP-1R immunoreactivity, consistent with dense

innervation by GLP-1 fibers in both rats and monkeys, but the ventromedial hypothalamic nucleus did not express GLP-1R. Brainstem areas involved in appetite regulation also expressed GLP-1R including the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus nerve. GLP-1R in the area postrema and nucleus of the solitary tract are accessible to circulating GLP-1 and these receptors may mediate the anorexic effects of peripheral GLP-1, including peripherally administered GLP-1 analogs. In rats, GLP-1Rs are not expressed on GLP-1 neurons of the nucleus of the solitary tract. GLP-1R expression also occurred in other brain areas affecting appetite and body weight including the lateral parabrachial nucleus, the amygdala, and the bed nucleus of the stria terminalis. The similar distribution of GLP-1R in brains from monkeys and rats indicate that peripherally administered GLP-1 agonists can affect food intake on several levels by:

1. activating the GLP-1R in the area postrema, an area of entrance into the brain.
2. activating GLP-1R in the nucleus of the solitary tract, which can directly bind circulating GLP-1 agonists.
3. affecting GLP-1R activation in the dorsal motor nucleus affecting the activity in the efferent vagus.
4. affecting neural transmission in the functional continuum linking the nucleus of the solitary tract with the lateral parabrachial nucleus with the paraventricular nucleus, amygdala, and bed nucleus of the stria terminalis.
5. directly affecting activities in 2 hypothalamic nuclei in appetite regulation including:
    a. the paraventricular nucleus and lateral hypothalamic area are the master regulators of endocrine, autonomic, and behavioral aspects of feeding.
    b. the arcuate nucleus containing parallel neurocircuitry regulating food intake including α-MSH/CART neurons and NPY/AgRP neurons.

Table 1 Comparison of the GLP-1R distribution in monkey and rat brain

Summary of presence "+" or absence "-" of GLP-1R in monkey (this study) and rat (1-4) brain regions measured by ISH, ISLB, and IHC. "+", "++", "+++", and "++++" indicate increasing signal intensities. N.A.= Not analysed.

Area	Monkey IHC (cell staining)	Monkey IHC (staining of fibers)	Monkey ISH	Monkey ISLB	Rat ISH	Rat ISLB
Cortex						
Prefrontal cortex	-	-	-	-	-	-
Parietal cortex	-	-	-	-	-	-
Temporal cortex	-	-	-	-	-	-
Occipital cortex	-	-	-	-	-	-
Basal Ganglia						
Nucleus accumbens	++	++	++	++	+	++
Caudate-Putamen	-	-	-	-	+	-
Striatum					++	++
Globus pallidus (internal)	+	+	+	+	N.A	N.A.
Globus pallidus (external)	-	-	-	-	N.A.	N.A.
Islands of Calleja	-	-	-	+++	-	++
Amygdala						
Amygdala (central nuclei)	++	++++	+++	+++	-	++
Amygdala (medial nuclei)	+	++	++	++	-	++
Bed nucleus of the stria terminalis (BST)	+++	+++	++	++	+	+
Septum						
Lateral septum	++	++	+	+	++	+++
Medial septum	++	++	+	+	+	+
Thalamus						
Anterodorsal nuclei	-	-	-	-	-	++
Centromedial nuclei	-		-	-	-	++
Laterodorsal nucleis	-	-	-	-	+	++
Paraventricular nucleus	+	+	++	+	++	+
Posterior nuclei	-	-	-	-	+++	++
Zona incerta	-	-	-	-	+	++
Hypothalamus						
Medial Preoptic area (MPO)	++	++	+	++	+++	+
Supraoptic nucleus (SON)	+++	+++	+++	++	+++	N.A.
Paraventricular nucleus (PVN)	+++	+++	+++	+++	++++	++
Lateral hypothalamic area (LHA)	+	+	++	+	+	++
Dorsomedial	++	++	++	++	+	++

Area	Monkey IHC (cell staining)	Monkey IHC (staining of fibers)	Monkey ISH	Monkey ISLB	Rat ISH	Rat ISLB
nucleus (DMH)						
Arcuate nucleus (Arc)	++++	++++	++++	++++	++++	+++
Median eminence	+++	+++	+	+	-	++++
Ventromedial nucleus (VMH)	-	-	-	-	-	+
Mesencephalon						
VTA	++	++	++	++	++	++
Substantia nigra	++	++	+	++	+	N.A.
Posterodorsal tegmental nucleus (PDTg)	++++	++++	++++	++++	+++	+++
Periaqueductal gray (PAG)	+	+++	+	++	++	+
Dorsal raphe (DR)	+	++	+	++	+	++
Pons						
Lateral reticular formation	++	+++	++	++	+	+
Parabrachial nucleus	++	+++	++	++	+	+
Locus coeruleus	+	+	+	++	+	N.A.
Medulla						
Nucleus of the solitary tract (NTS	++++	++++	++	++++	++++	++++
Area postrema (AP)	++++	++++	++++	++++	++++	++++
Dorsal motor nucleus of the vagus (DMX)	++	++++	+	++++	N.A.	N.A.
A1/C1	-	++	+	++	N.A.	N.A.
Gigantocellular nucleus (GI)	+	++	+	++	N.A.	N.A.
Inferior olive	-	++	+	++	+++	+++
Spinal cord						
Dorsal horn	-	++++	-	++++	N.A.	N.A.
Cerebellum (lobule 9-10)						
Medial cerebellar nuclei	+++	+++	+++	+++	N.A.	N.A.

[P32 – 34]

Study Title: Acute effects of liraglutide on cFos expression in the rat brain (report Kdhl090408)

Key Study Findings

- A single subcutaneous dose of 0.1 mg/kg liraglutide reduced food consumption of male rats from 2 hours and 15 minutes to 4 hours after dosing.
- Feeding increased the number of cFos immunoreactive neurons and the proportion of cFos(+) GLP-1 neurons in the nucleus of the solitary tract (brainstem), increased the number of cFos(+)neurons and the proportion of cFos(+) CART neurons in the arcuate nucleus (forebrain), and increased the number of cFos(+) neurons in the central amygdala (forebrain) and paraventricular nucleus (forebrain). Feeding had no effect on the number of

cFos(+) neurons or the proportion of cFos(+) dopaminergic and/or adrenergic neurons in the area postrema (brainstem) and no effect on the number of cFos(+) neurons in the lateral parabrachial nucleus.

- Liraglutide affected cFos expression in the brain areas regulating food intake and body weight including brainstem area postrema and nucleus of the solitary tract, the lateral parabrachial nucleus and amygdala, and the hypothalamic arcuate nucleus. Four hours after dosing, liraglutide:
  - increased the number of cFos(+) neurons and the proportion of cFos(+) dopaminergic and/or adrenergic neurons in the area postrema in fed and fasted rats, but with no interaction between feeding and liraglutide treatment. In fasted rats, some neurons in the area postrema activated by liraglutide were not adrenergic and/or dopaminergic.
  - increased the number of cFos(+) neurons in the nucleus of the solitary tract in fed and fasted rats, but decreased the proportion of cFos(+) GLP-1 neurons in the nucleus of the solitary tract of fed rats without affecting the proportion of cFos(+) GLP-1 neurons in fasting rats. In the nucleus of the solitary tract, liraglutide activated non-GLP-1 neuron in fed rats and fasted rats and reduced activation of GLP-1 neurons in the nucleus of the solitary tract of fed rats.
  - decreased the number of cFos(+) neurons and the proportion of cFos(+) CART neurons in the arcuate nucleus of fed rats, but not in fasted rats.
  - increased the number of cFos(+) neurons in the lateral parabrachial nucleus of fed and fasted rats and in the central amygdala of fed rats, but liraglutide had no effect on the number of cFos(+) neurons in the central amygdala of fasted rats or in the paraventricular nucleus of fed or fasted rats.
- Study deficiencies were:
  - the specificity of reagents used for immunohistochemical analysis was not reported or evaluated.

Summary

To identify brain areas activated by liraglutide in rats, cFos expression (a proto-oncogene that is an immediate early gene with increased expression in activated neurons) was evaluated in brain from fed and unfed male Sprague Dawley rats (8/group/fed state, 8 weeks old, ~250 g body weight) 4 hours after a single subcutaneous dose of vehicle or 0.1 mg/kg liraglutide. In the first part of the study, fed rats were administered a single subcutaneous dose of vehicle (0.025% rat albumin in phosphate buffered saline) or 0.1 mg/kg liraglutide (in vehicle) and food intake was monitored for 4 hours after dosing. In the second part of the study, rats were fasted for 1 hour prior to dosing with vehicle or 0.1 mg/kg/day liraglutide, and food was withheld for 4 hours after dosing. Four hours after dosing, fed or unfed rats were anesthetized (fentanyl, fluanisone, and midazolam) and terminated by transcardial perfusion with heparinized saline followed by 4% paraformaldehyde. Brains were fixed in 4% formaldehyde overnight, then for 2 days in 30% sucrose, sectioned (40 μm thick coronal sections), stored in 50 mM KCl in phosphate buffered saline (pH 7.4), and processed for immunohistochemical analysis for cFos (to identify activated neurons), cocaine and amphetamine regulated transcript (CART, to identify CART-expressing neurons), glucagon-like peptide 2 (GLP-2, to identify neurons expressing GLP-1), and tyrosine hydroxylase (TH, to identify dopaminergic / adrenergic neurons). cFos immunohistochemical staining used a primary rabbit anti-cFos antibody a secondary biotinylated donkey anti-rabbit antibody, and ABC-streptavidin horse radish peroxidase (HRP) with color developed using diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. cFos immunoreactivity was quantified in specific brain areas by counting the number of cFos(+) cell nuclei using a computerized image analysis system. Brain sections immunohistochemically stained for both GLP-2 and TH used mouse monoclonal antibodies directed against GLP-2 and TH, respectively, and the applicant contends tyramide amplification permits the use of the 2

primary mouse antibodies. For triple staining, brain sections were incubated with rabbit anti-cFos and mouse anti-GLP-2 antibodies overnight followed by incubation with biotinylated donkey anti-rabbit antibody and ABC-streptavidin HRP to label cFos, then incubated with biotinylated tyramide and hydrogen peroxide followed by incubation with streptavidin-Alexa546 (labeled cFos). The slide was incubated with 1% hydrogen peroxide to destroy the remaining HRP activity prior to incubation with HRP-conjugated donkey anti-mouse antibody to label the GLP-2 antibody and incubated with FITC-labeled tyramine. Finally, brain sections were incubated with a mouse anti-TH antibody followed by a secondary Cy5-coupled donkey anti-mouse antibody to label TH. Brain sections were processed for double immunohistochemical analysis of cFos and CART. Triple immunohistochemical analysis for cFos, TRH, and CART was performed using a similar tyramide amplification procedure described for the triple staining for cFos, GLP-2, and TH. For colocalization, slides were examined by fluorescence microscopy and semi-quantitative analysis was performed using 6 μm thick optical sections except the number of cells co-expressing cFos was scored manually (blinded to treatment).

Figure 1 shows food intake in fed rats was reduced from 135 to 240 minutes after dosing.

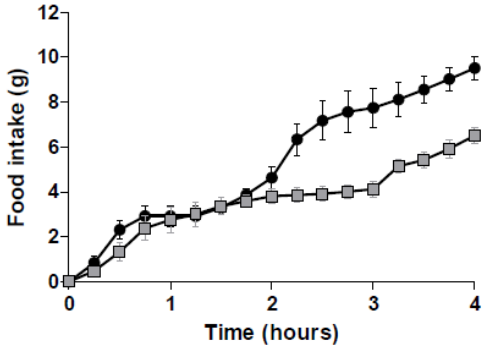


Figure 1 Food intake in rats terminated at 4 hours
Data= mean ± SEM. Black circles: vehicle; grey circles: liraglutide 100 μg/kg s.c.

[P15]

The effects of food consumption, liraglutide treatment, and both food consumption and liraglutide treatment on the number of cFos immunoreactive neurons in specific brain regions are summarized in Table 3 (below). Feeding increased the number of cFos immunoreactive neurons in the nucleus of the solitary tract (NTS), central amygdala (CeA), arcuate nucleus (ARC), and hypothalamic paraventricular nucleus (PVN), but not in the area postrema (AP) or lateral parabrachial nucleus (lPBN).

Table 3 Summary of changes in cFos-immunoreactivity (2-way ANOVA)

cFos-ir in	Affected by liraglutide p-values (F-values)	Affected by feeding p-values (F-values)	Interaction p-values (F-values)
AP	Increased p<0.0001 (F= 35.53)	No effect p=0. 35(F= 0.91)	No interaction p=0.15 (F= 2.16)
NTS	Increased p<0.0001 (F= 29.58)	Increased p<0.028 (F= 5.37)	No interaction p=0.42 (F= 0.67)
lPBN	Increased p<0.0001 (F= 58.49)	No effect p=0.098 (F= 2.93)	No interaction p=0.066 (F= 3.67)
CeA	Increased p<0.0001 (F= 25.0)	Increased p=0.033 (F= 5.04)	Interaction p=0.045 (F= 4.41)
ARC	Decreased p=0.0077 (F= 8.28)	Increased p=0.019 (F= 6.24)	No interaction p=0.74 (F= 0.11)
PVN	No effect p=0.58 (F= 0.31)	Increased p=0.035 (F= 4.92)	No interaction p=0.93 (F= 0.006)

[P26]

Liraglutide increased the number of cFos immunoreactive neurons in the AP (Figure 3), NTS (Figure 4), and lPBN (Figure 5) in both fed and infed rats and in the CeA of fed rats, but not unfed rats (Figure 6). Liraglutide decreased by the number of cFOS immunoreactive neurons in the ARC of fed rats, but not unfed rats (Figure 7), but it did not affect the number of cFOS immunoreactive neurons in in the hypothalamic PVN (Figure 8). Both feeding and liraglutide treatment interacted to further increase the number of cFos immunoreactive neurons in the CeA (Table 3, above).

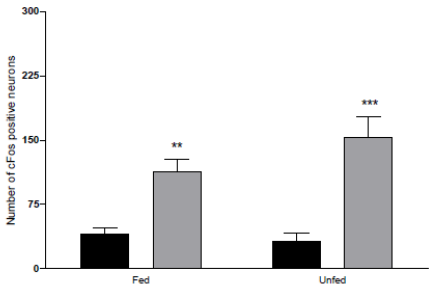


Figure 3 cFos immunoreactivity in the area postrema (AP). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p= 0.0032); Liraglutide versus Vehicle unfed (p<0.0001)

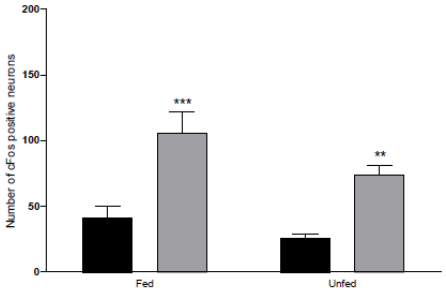


Figure 4 cFos immunoreactivity in the nucleus of the solitary tract (NTS). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p= 0.0001); Liraglutide versus Vehicle unfed (p= 0.0034)

[P18]

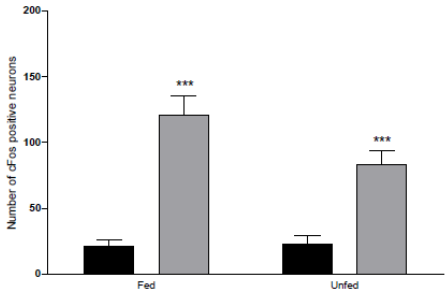


Figure 5 cFos immunoreativity in the lateral parabrachial nucleus (lPBN). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p<0.0001); Liraglutide versus Vehicle unfed (p= 0.0005)

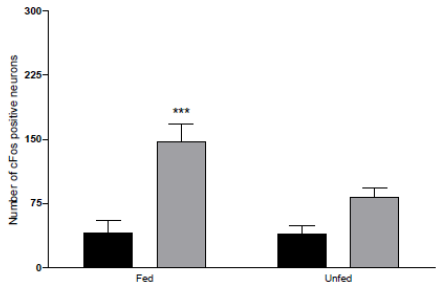


Figure 6 cFos immunoreactivity in the central amygdala (CeA). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p<0.0001)

[P19]

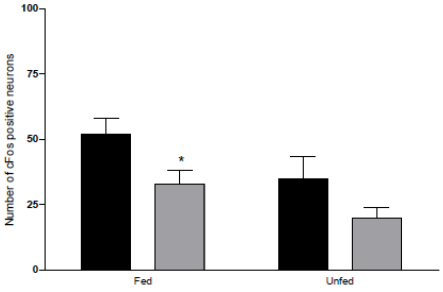


Figure 7 cFos immunoreactivity in the arcuate nucleus (ARC). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p=0.029)

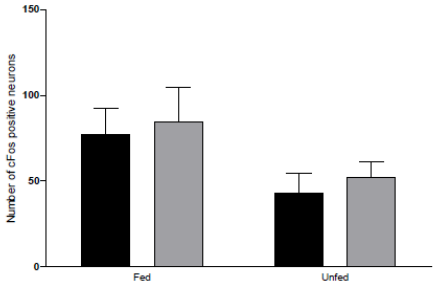


Figure 8 cFos immunoreactivity in the hypothalamic paraventricular nucleus (PVN). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8

[P20]

The neurochemical phenotype of cFos immunoreactive neurons was investigated by conccurrent immunonhistochemical staining for cFos and tyrosine hydroxylase (to identify dopaminergic and adrenergic neurons in the AP), CART (to identify neurons expressing CART in the ARC), and GLP-2 (to identify neurons expressing GLP-1 in the NTS). Table 4 summarizes results showing the effects of feeding and liraglutide treatment on the proportion of cFos(+)TH immunoreactive neurons in the AP, the proportion of cFOS(+) GLP-2 immunoreactive neurons in the NTS, and the proportion of cFos(+) CART immunoreactive neurons in the ARC. Feeding had no effect on the proportion of cFos(+) TH immunoreactive neurons in the AP, but feeding increased the proportion of cFos(+)GLP-2 immunoreactive neurons in the NTS and cFos(+) CART immunoreactive neurons in the ARC. Liraglutide increased the proportion of cFos(+)TH-immunoreactive neurons in the AP in both fed and unfed rats, and decreased the proportion of cFos(+) GLP-2 immunoreactive neuron in the NTS and cFos(+) CART immunoreactive neurons in the ARC in fed rats, but not in unfed rats (Figures 10, 11, and 12, respectively, below).

Table 4 Summary of changes in cFos-ir in specific neurons (2-way ANOVA)

cFos-ir in	Affected by liraglutide p-values (F-values)	Affected by feeding p-values (F-values)	Interaction p-values (F-values)
TH-ir in AP	Increased p=0.0002 (F= 18.24)	No effect p=0.55 (F= 0.36)	No interaction p=0.646 (F= 0.21)
GLP-2-ir in NTS	Decreased p=0.020 (F= 6.12)	Increased p<0.0001 (F= 107.2)	Interaction p=0.017 (F= 6.50)
CART-ir in ARC	No effect p=0.080 (F= 8.17)	Increased p<0.0001 (F= 23.94)	No interaction p=0.06 (F= 3.81)

[P27]

Figure 9 shows colocalization of cFos immunoreactive and TH immunoreactive neurons in the AP (white arrows). In the AP of liraglutide-treated fed and unfed rats, approximately 20% of TH immunoreactive neurons were also cFos immunoreactive, but TH and cFos immunoreactivities were not colocalized in AP neurons of vehicle-treated fed or unfed rats (Figure 10, below).

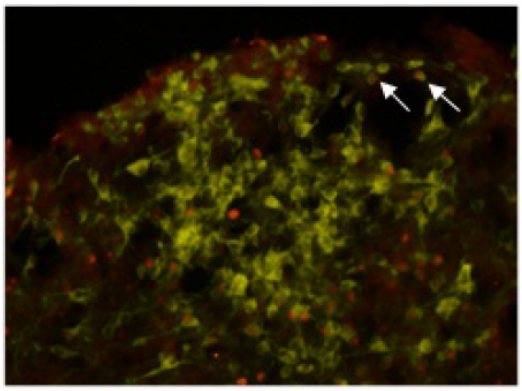


Figure 9 cFos-ir (red) and TH-ir (green) neurons in the AP. Arrows point s to cFos activated neurons

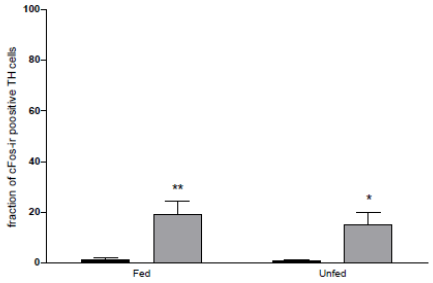


Figure 10 Fraction of tyrosine hydroxylase (TH)-ir cells containing cFos in the area postrema (AP). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p=0.0025); Liraglutide versus Vehicle unfed (p=0.012)

[P21, 22]

In the NTS, fasting significantly reduced the proportion of cFos(+) GLP-1 neurons (GLP-2 immunoreactive) (Figures 11 and 12, below), and consistent with its effect to reduce food consumption, liraglutide significantly reduced the proportion of cFos(+) GLP-1 neurons in fed rats (Figure 12).

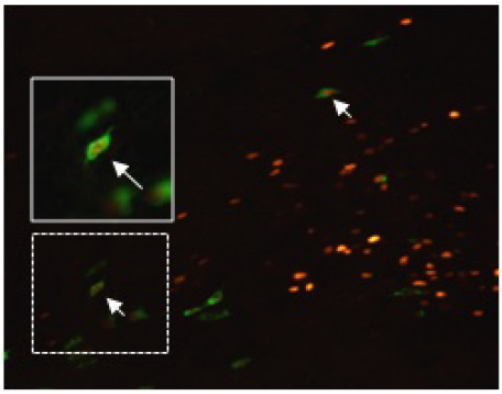


Figure 11 cFos-ir (red) and GLP-2-ir (green) neurons in the NTS. Arrows points to cFos activated neurons. Squares = high magnification photographs.

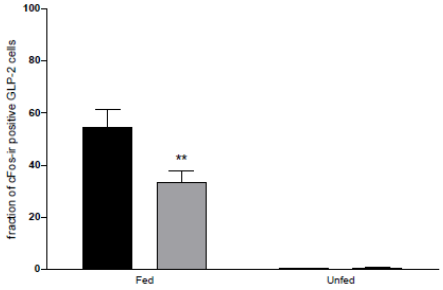


Figure 12 Fraction of GLP-2-ir cells containing cFos in nucleus of the solitary tract (NTS). Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p=0.0017)

[P23, 24]

In the hypothalamic ARC, fasting reduced the number of cFos(+) CART neurons (Figures 13 and 14), and liraglutide reduced the proportion of cFos(+) CART neurons in fed rats without significantly affecting the proportion of cFos(+) CART neurons in unfed rats (Figure 14).

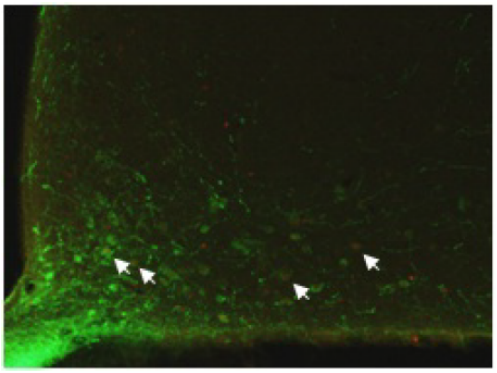


Figure 13 cFos-ir (red) and CART-ir (green) neurons in the ARC. Arrows point s to cFos activated neurons.

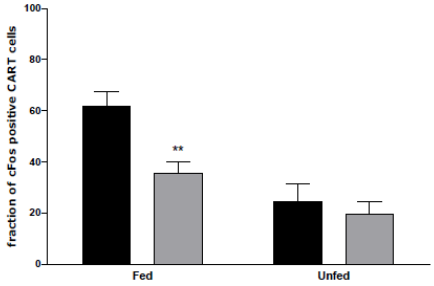


Figure 14 Fraction of CART-ir cells containing cFos in the ARC. Black bars = vehicle; Grey bars = liraglutide (100 µg/kg). Data are mean ± SEM, n=8. Liraglutide versus Vehicle fed (p=0.0020)

[P25, 26]

Study Title: Liraglutide effects on body weight: The role of vagal afferents (report KLYK130301)

Key Study Findings

- Vagal deafferentiation had no substantive effect food consumption, water consumption, or body weight gain in rats.
- Vagal deafferentiation was confirmed by a functional test (loss of CCK-induced increased food intake) and histologically (fluorogold, a retrograde tracer confirming subdiaphragmatic vagal afferents and efferents were severed and WGA, a anterograde and retrograde tracer confirming left vagal afferent rootlets were severed).
- The effects of liraglutide to slow gastric emptying, transiently reduce water intake, reduce food consumption, and decrease body weight gain were not substantially mediated by vagal afferent innervation in rats.

Summary

The effects of liraglutide on gastric emptying, food consumption, and body weight gain were evaluated in sham-operated and subdiaphragmatic vagal deafferentiated (SDA) male Sprague Dawley rats. For subdiaphragmatic vagal deafferentiation, rats were anesthetized with 63 mg/kg ketamine and 9.4 mg/kg xylazine and the left vagal afferent rootlets were severed at the site of attachment to the brainstem (contain afferents from ventral gastric vagus accessory celiac nerve, and the hepatic vagus supplying the liver and proximal duodenum) without affecting efferent rootlets. A subdiaphragmatic vagotomy of the dorsal vagal trunk (near the stomach and esophagus) disconnected all vagal afferents and efferents supplying the dorsal gastric and celiac branches. For the sham operation, the left cervical vagus was exposed where it penetrates the foramen of the skull and a midline laparotomy exposed the subdiaphragmatic vagal trunk, but the nerves remained intact. The presence and absence of vagotomy was confirmed by evaluating the effects of cholecystokinin (CCK) on food intake and histological verification by evaluating staining of vagal nerves after intraperitoneal injection of fluorogold and injection of wheat germ agglutinin (WGA) in the nodose ganglion where the afferent rootlets were severed. Treatment groups are summarized in Table 1 (below) and an outline of the study is shown in Figure 2.1 (below). Sham-operated rats (groups 1 and 3) and SDA rats (groups 2 and 4) were subcutaneously injected with vehicle (0.1% bovine serum albumin in phosphate buffered saline, groups 1 and 2) or 0.2 mg/kg liraglutide (groups 3 and 4) twice a day for 14 days.

Table 1 Treatment groups, dose, dose volume and group size for the main study

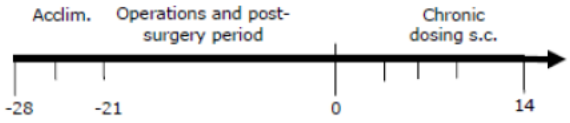
Group	Treatment	Dose	Dose volume, s.c.	Group size per day
1) Sham	Vehicle	-	2 ml/kg	n=6
2) SDA	Vehicle	-	2 ml/kg	n=8
3) Sham	Liraglutide*	200 µg/kg BID**	2 ml/kg	n=7
4) SDA	Liraglutide*	200 µg/kg BID**	2 ml/kg	n=8

* For test of gastric emptying day 0, liraglutide was dosed intraveneously 100 µg/kg (2 ml/kg)
**BID: twice daily
Vehicle: Lonza DPBS-buffer (batch 9MB145)

[P12]

2.1 Study outline

Experimental study outline: vagal de-afferentiation



Animal and operational procedure

45 male SPD rats (8 weeks of age) were purchased from (b) (4) and acclimatized for at least 5 days before operational procedures. 12 rats were used for surgical optimization.

16 animals were successfully selective de-afferentiated and 13 rats were successfully sham operated. Following a post-surgery period rats were randomized into the following groups and dosed for 14 days:

Group 1: Vehicle, sham (s.c. bi-daily, n=6)

Group 2: Vehicle, SDA (s.c. bi-daily, n=8)

Group 3: Liraglutide, sham (s.c. bi-daily, n= 7)

Group 4: Liraglutide, SDA (s.c. bi-daily, n 8)

Body weight, food intake and water intake (day 0-14)

Food intake, water intake and body weight was monitored daily throughout the 14 day study period.

Acute gastric emptying (day 0)

A single dose of acetaminophen was given on day 0. Plasma was collected at time point, t= 0, 15, 30, 60, 120 and 180.

Termination

Two tests were conducted in order to verify the SDA procedure: 1) a functional test assessing the effect of vagus mediated CCK signalling, and 2) a histological test based on specific afferent and efferent tracers.

[P10]

Vagotomy was confirmed by the absence of a reduction in food intake by CCK. Dose groups are summarized in Table 2 (below). Overnight fasted sham operated and SDA rats were administered a single intraperitoneal dose of vehicle (phosphate-buffered saline) or 8 mcg/kg CCK (1 mL/kg dose volume, CCK dissolve in 0.1% BSA in PBS) on day -1, and 15 minutes after the injection, food intake was monitored for up to 120 minutes after food presentation. On day 2, the study was repeated by administering CCK to rats previously treaetd with vehicle and vice versa and rats were euthanized by CO_2 inhalation and decapitation after completing the study.

Table 2 Dosing regimen for verification of intact vagotomy

	Day 0	Day 2	Dose volume	Group size per day
1) SDA	Vehicle (saline)	CCK, 8 µg/kg	1 ml/kg i.p.*	n=4
2) SDA	CCK, 8 µg/kg	Vehicle (saline)	1 ml/kg i.p.	n=4
3) Sham	Vehicle (saline)	CCK, 8 µg/kg	1 ml/kg i.p.	n=3
4) Sham	CCK, 8 µg/kg	Vehicle (saline)	1 ml/kg i.p.	n=3

* i.p.: Intraperitoneally

[P13]

Figure 7 shows food and water intake 15 and 30 minutes after vehicle or CCK injection in sham operated and SDA rats. In vehicle groups, food intake 30 minutes after dosing was lower in SDA rats compared to sham operated rats. In sham operated rats, CCK decreased food intake 15 and 30 minutes after dosing and decreased water intake 15 minutes after dosing. In SDA rats, CCK had no effect on food or water intake for up to 30 minutes after dosing.

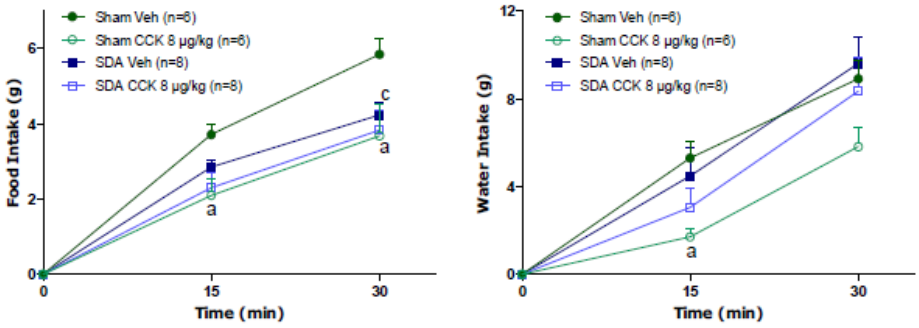


Figure 7 Accumulated food and water intake 15 and 30 minutes after CCK injection i.p. Analysis by two-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham CCK vs. Sham Veh
[c]p<0.05 SDA Veh vs. Sham Veh

[P22]

For histological veritification of vagotomy, SDA or sham operated rats (3/group) were intraperitoneally injected with 3 mg/kg fluorogold (1 mL/kg dose volume, saline vehicle, Fluorochrome, Denver, CO), a retrograde tracer, and injected into the nodose ganglion (where the afferent roots were severed near the brain) with1 µL 10% WGA (in 0.1% BSA in PBS), a retrograde and anterograde tracer. Three days after tracer injection, rats were anesthetized and sacrificed by perfusion fixation with 4% paraformaldehyde, and sections of brain (40 µm thick) were processed for immunohistochemical analysis of fluorogold and WGA by fluorescence microscopy. Figure 8 shows WGA immunoreactivity in the left dorsal motor nucleus in both sham operated and SDA rats while afferent fibers were present in sham operated rats, but not in SDA rats.

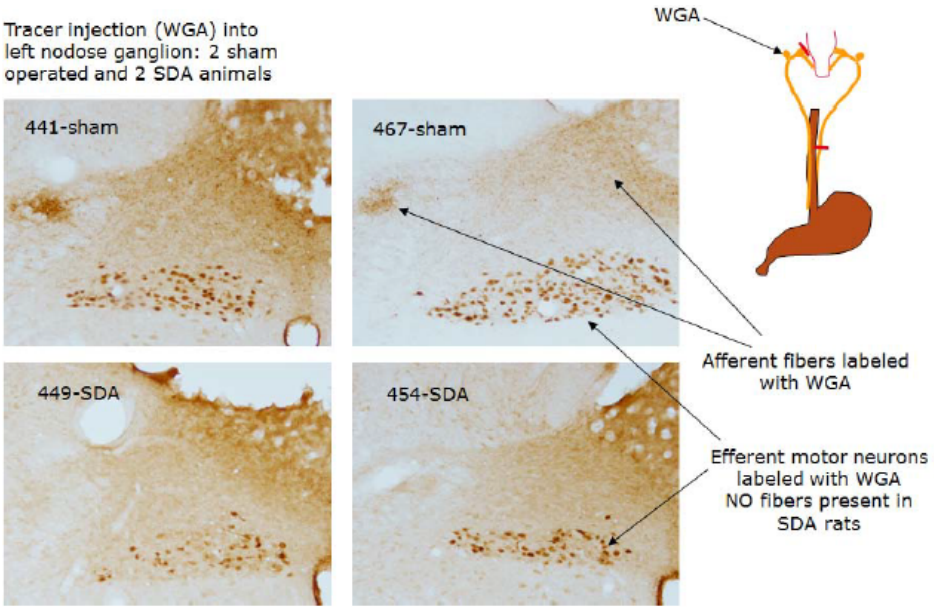


Figure 8 **Brain stem sections showing both afferent and efferent WGA labelling in sham animals (# 441 and 467) whereas only efferent labelling was seen in SDA animals (# 449 and 454). WGA was injected into the left nodose ganglion.**

[P23]

Figure 9 shows the SDA procedure decreased fluorogold labeling in the gastrointestinal part of the dorsal motor nucleus confirming vagotomy of dorsal gastric and celiac branches.

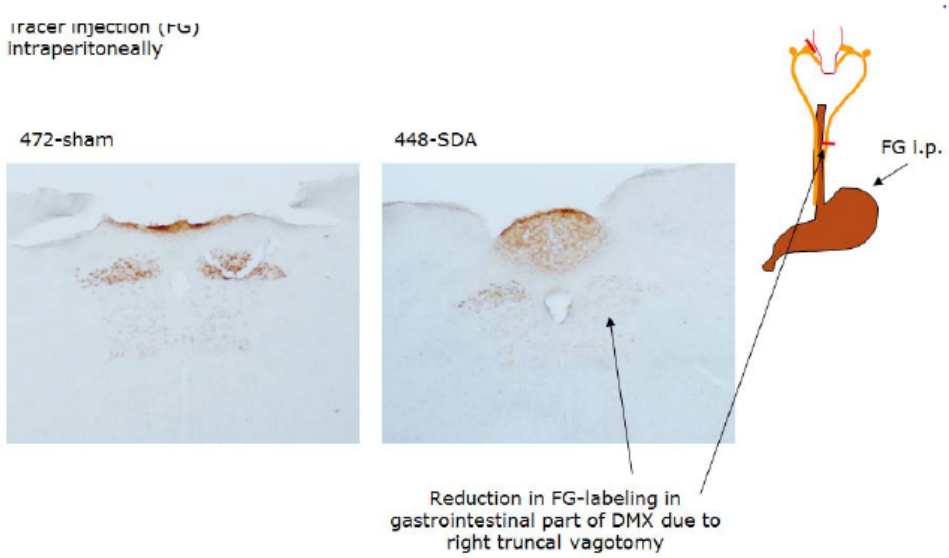


Figure 9 **Brain stem sections showing reduced FG labelling in truncal lesioned SDA animals (no 448) compared to sham (no 472). FG was injected i.p.**

[P24]

An acute gastric emptying study was performed on the first day of dosing. For this study, rats were offered 50% of their normal food consumption 24 hours prior to being administered a single iv dose of vehicle or 0.1 mg/kg liraglutide (2 mL/kg) followed 15 minutes later by a single

oral dose of 160 mg/kg acetaminophen (10 mL/kg of 16 mg/mL acetaminophen suspension) and blood levels of acetaminophen were evaluated for up to 180 minutes after acetaminophen dosing. Liraglutide reduced plasma acetaminophen $AUC_{0\text{-}180min}$ indicating liraglutide slowed gastric emptying in both sham and SDA rats (Figure 6, below).

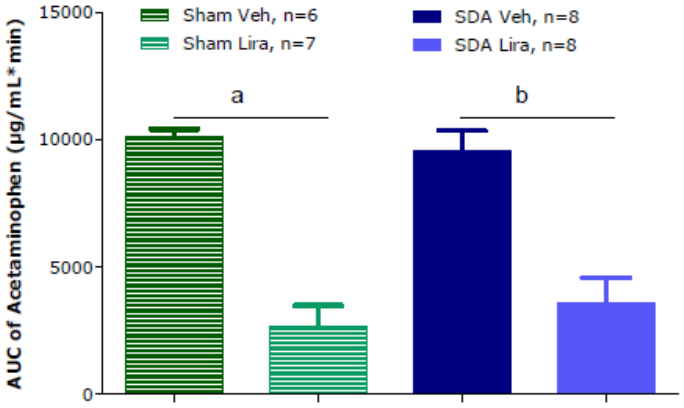


Figure 6 AUC of acetaminophen plasma concentration in Figure 5
Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 SDA Lira vs. SDA Veh

[P21]

Vagotomy did not affect initial body weight in rats, but both sham operated rats and SDA rats were sensitive to liraglutide-related decreased body weight gain. After the gastric emptying study, the dose of liraglutide was increased to 0.2 mg/kg/injection liraglutide subcutaenously injected twice a day. On day -1 in vehicle only groups, body weight of sham operated rats and SDA rats were similar (Table 3, below). Group mean body weight was increased during the treatment period in vehicle-treated sham rats and SDA rats and liraglutide-treated sham rats, but not in liraglutide-treated SDA rats (Table 3 and Figures 1 and 2, below). In vehicle treated groups, body weight and body weight gain in SDA rats was similar to sham-operated rats throughout the treatment period. Compared to their respective vehicle groups on day 14, liraglutide decreased body weight and body weight gain of sham operated rats and SDA rats. In sham operated rats, body weight gain was 88.0% lower and body weight was 9.3% lower in the liraglutide group compared to the vehicle group. In SDA rats, body weight gain was 100% lower and body weight was 9.7% lower in the liraglutide group compared to the vehicle group. Compared to vehicle treated groups, body weight change as a percentage of initial body weight was significantly lower in liraglutide treated sham operated rats (compared to vehicle treated sham operated rats) and SDA rats (compared to vehicle treated SDA rats) (Figure 2, below).

Table 3 Body weight (start-end) and delta body weight

	Day -1	Day 14	Delta (day -1 to 14)
Sham Veh, n=6	389.3 ± 23.6	440.1 ± 38.2	50.8 ± 17.7
Sham Lira, n=7	392.9 ± 24.9	399.0 ± 22.5	6.1 ± 10.0 **a**
SDA Veh, n=8	383.6 ± 22.8	428.5 ± 24.7	44.9 ± 9.9
SDA Lira, n=8	386.9 ± 29.8	386.8 ± 36.7 **b**	-0.2 ± 12.1 **b**

Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 SDA Lira vs. SDA Veh

[P18]

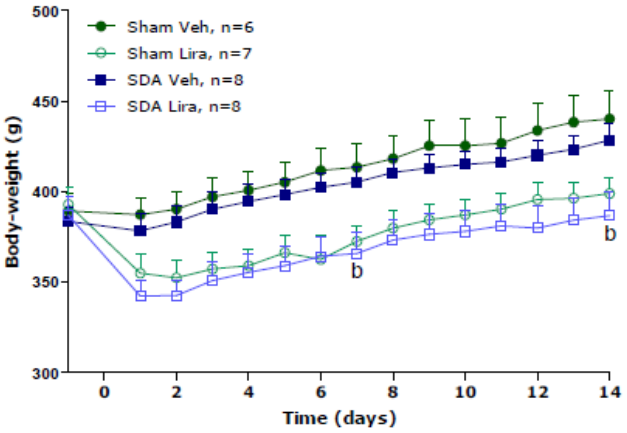


Figure 1 Body weight
Analysis by two-way ANOVA and post hoc Bonferroni (day 7 and 14). Data are mean ± SEM.
[b]p<0.05 SDA Lira vs. SDA Veh

[P17]

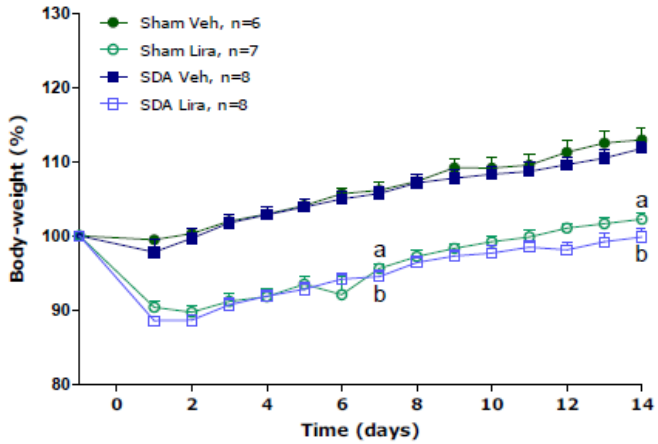


Figure 2 Body weight change in percentage of day -1
Analysis by two-way ANOVA and post hoc Bonferroni (day 7 and 14). Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 SDA Lira vs. SDA Veh

[P18]

Vagotomy had no effect on food intake in rats and liraglutide transiently lowered food consumption in both sham operated and SDA rats (Figure 3, below). In vehicle treated groups, food intake was similar in SDA rats compared to sham operated rats throughout the treatment period. Compared to vehicle treated groups, food intake was decreased during the first 6 days of liraglutide treatment in both sham-operated and SDA rats and during days 7 – 9 and 12 in liraglutide-treated SDA rats

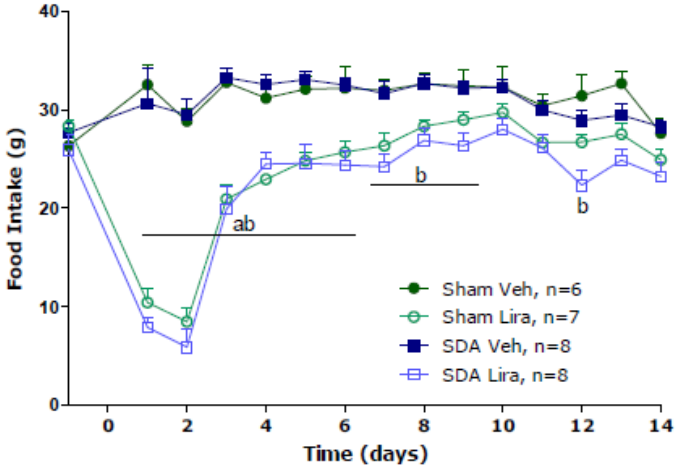


Figure 3 Food intake
Analysis by two-way ANOVA and post hoc Bonferroni. Statistical analysis performed all days except day 11 and 14 where one data point was missing. Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 SDA Lira vs. SDA Veh

[P19]

Vagotomy had no effect on water intake, but liraglutide transiently decreased water intake in both sham operated and SDA rats (Figure 4, below). Compared to vehicle groups, liraglutide transitently decreased water intake in sham operated rats (days 1 and 2, compared to vehicle treated sham operated rats) and in SDA rats (days 1 and 2, compared to vehicle treated SDA rats).

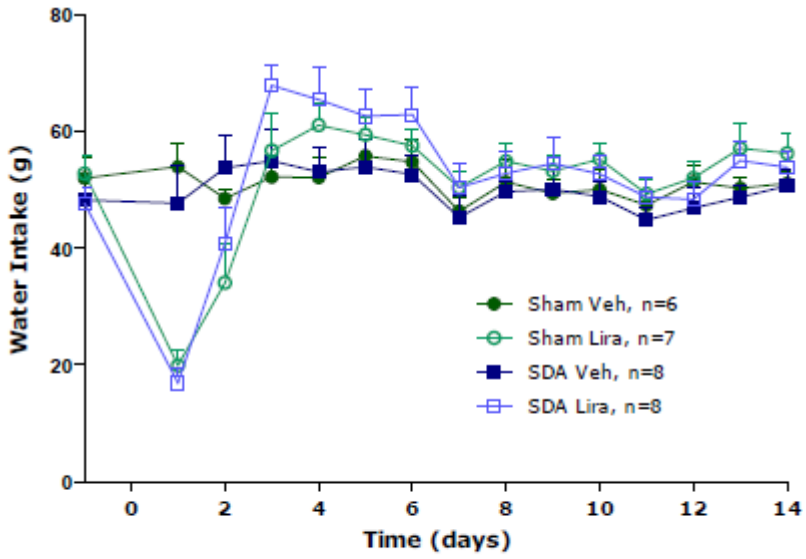


Figure 4 Water intake
Data are mean ± SEM.
[P20]

Study Title: The role of area postrema in mediating liraglutide effects on food intake and body weight (report KLYK130302)

Key Study Findings

- Ablation of the brainstem area postrema decreased food consumption and body weight gain in rats.
- The effects of liraglutide to slow gastric emptying were not mediated by the area postrema in rats.
- The effects of liraglutide to transiently reduce water consumption and food consumption and to reduce body weight gain are not mediated by the area postrema in rats.

Summary

The effects of liraglutide on body weight and food consumption were evaluated in sham-operated and area postrema-ablated male Sprague Dawley rats. Treatment groups are summarized in Table 1 (below) and an outline of the study is shown in Figure 2.1 (below). Rats anesthetized with 63 mg/kg ketamine and 9.4 mg/kg xylazine underwent an operation to ablate the brainstem area postrema by aspiration with a blunt 23 gauge needle. For the sham operation, the brain medulla was exposed, but not lesioned. The presence and absence of area postrema ablation was confirmed by microscopic examination of Thionin-stained brain tissues slices from all rats at the end of the study. Sham-operated rats (groups 1 and 3) and AP-ablated rats (groups 2 and 4) were subcutaneously injected with vehicle (0.1% bovine serum albumin in phosphate buffered saline, groups 1 and 2) or 0.2 mg/kg/day liraglutide (groups 3 and 4) twice a day for 21 days.

Table 1 Treatment groups, dose, dose volume and group size for the main study

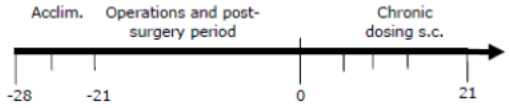
Group	Treatment	Dose	Dose volume, s.c.[x]	Group size**
1) Sham	Vehicle	-	2 ml/kg	n=7 (n=6)
2) APx	Vehicle	-	2 ml/kg	n=11 (n=10)
3) Sham	Liraglutide*	200 µg/kg BID[xx]	2 ml/kg	n=6 (n=6)
4) APx	Liraglutide*	200 µg/kg BID[xx]	2 ml/kg	n=13 (n=10)

* For test of gastric emptying day 0, liraglutide was dosed intravenously (i.v.) 100 µg/kg (2 ml/kg)
** Group size for chronic study is listed first and group size for gastric emptying is in parenthesis
[x]s.c.: Subcutaneously
[xx]BID: Twice daily
Vehicle: Lonza DPBS-buffer (batch 9MB145)

[P11]

2.1 Study Outline

Experimental study outline: area postrema ablation



Animal and operational procedure

50 male SPD rats (8 weeks of age) were purchased from (b) (4) and acclimatized for at least 5 days before operational procedures.

24 animals underwent a successfully Area Postrema lesion (APx), 13 rats were successfully sham operated. Following a post-surgery period rats were randomized into the following groups:

Group 1: Vehicle, sham (s.c. bi-daily, n=7)

Group 2: Vehicle, APx (s.c. bi-daily, n=11)

Group 3: Liraglutide, sham (s.c. bi-daily, n=6)

Group 4: Liraglutide, APx (s.c. bi-daily, n=13)

Body weight, food intake and water intake (day 0-21)

Food intake, water intake and body weight was monitored daily throughout the 21 day study period.

Acute gastric emptying (day 0)

A single dose of acetaminophen was given at day 0. Plasma was collected at time points t= 0, 15, 30, 60, 120, and 180.

Termination

Animals were euthanized by decapitation under CO_2/O_2 anaesthesia. The brains were removed and frozen. In order to verify the APx procedure all ablated rats were histologically examined. Forebrains were stored for optional in situ hybridization.

[P9]

An acute gastric emptying study was performed on the first day of dosing. For this study, rats were offered 50% of their normal food consumption 24 hours prior to being administered a single subcutaenous dose of vehicle or 0.1 mg/kg liraglutide (2 mL/kg) followed 15 minutes later by a single oral dose of 160 mg/kg acetaminophen (16 mg/mL acetaminophen suspension) and blood levels of acetaminophen were evaluated for up to 180 minutes after acetaminophen dosing. Liraglutide reduced acetaminophen $AUC_{0\text{-}180min}$ indicating liraglutide slowed gastric emptying in both sham and AP-ablated rats (Figure 7, below).

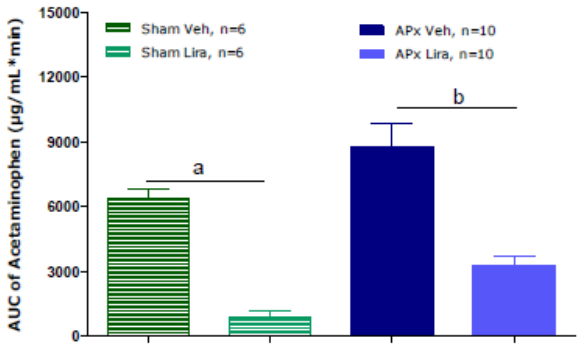


Figure 7 AUC of acetaminophen plasma concentration depicted from Figure 6
Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 APx Lira vs. APx Veh

[P20]

AP ablation decreased body weight gain in rats, but did not ameliorate liraglutide-induced decreased body weight gain. After the gastric emptying study, the dose of liraglutide was increased to 0.2 mg/kg/injection liraglutide twice a day. On day -1 in vehicle only groups, body weight of AP-ablated rats (group 2) was significantly lower than sham operated rats (group 1) (Table 2, below). All rats gained wieght during the 21 day treatment period (Table 2 and Figures 2 and 3, below). Ablation of the area postrema reduced body weight and body weight gain in rats. In vehicle treated rats, body weight and body weight gain in AP-ablated rats was significantly lower than sham-operated rats on study days 7, 14, and 21 with 14.4% lower body weight and 36.7% lower body weight gain in AP-lesioned rats compared to sham operated rats on day 21. Liraglutide decreased body weight and body weight gain of sham operated and AP-ablated rats rats. In sham operated rats, body weight gain was 43.3% lower and body weight was 7.3% lower in the liraglutide group compared to the vehicle group. In AP-ablated rats, body weight was significantly lower in the liraglutide group compared to the vehicle group on days 7, 14, and 21 (Figure 2, below) and on day 21, body weight was 5.6% lower and body weight gain was 40.1% lower in the liraglutide group. Compared to vehicle treated groups, body weight change as a percentage of the initial body weight was significantly lower in liraglutide treated sham operated rats (compared to vehicle treated sham operated rats) and AP-ablated rats (compared to vehicle treated AP-ablated rats) (Figure 3, below).

Table 2 Body weight (start-end) and delta body weight

	Day -1	Day 21	Delta (day -1 to 21)
Sham Veh (n=7)	360.8 ± 15.2	428.9 ± 22.0	68.1 ± 14.9
Sham Lira (n=6)	359.2 ± 12.5	397.8 ± 15.7 **a**	38.6 ± 6.4 **a**
APx Veh (n=11)	325.6 ± 14.8 **c**	367.3 ± 17.9 **c**	43.1 ± 13.9 **c**
APx Lira (n=13)	320.8 ± 15.0	346.6 ± 20.2	25.8 ± 13.1 **b**

Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 APx Lira vs. APx Veh
[c]p<0.05 APx Veh vs. Sham Veh

[P17]

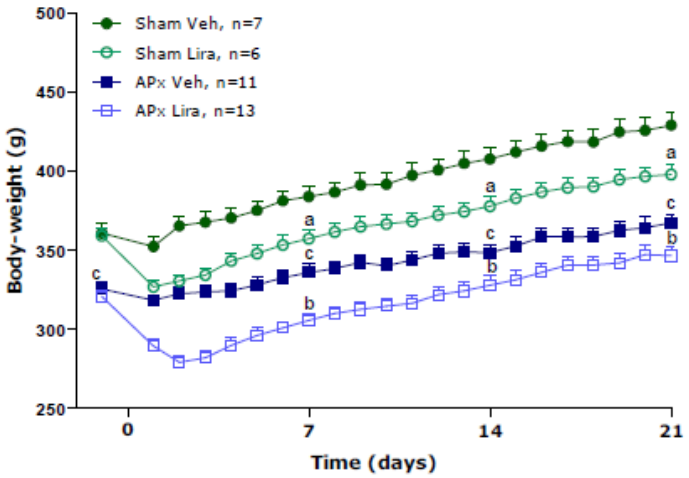


Figure 2 Body weight
Analysis by two-way ANOVA post hoc Bonfferoni (day -1, 7, 14 and 21)
Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 APx Lira vs. APx Veh
[c]p<0.05 APx Veh vs. Sham Veh

[P16]

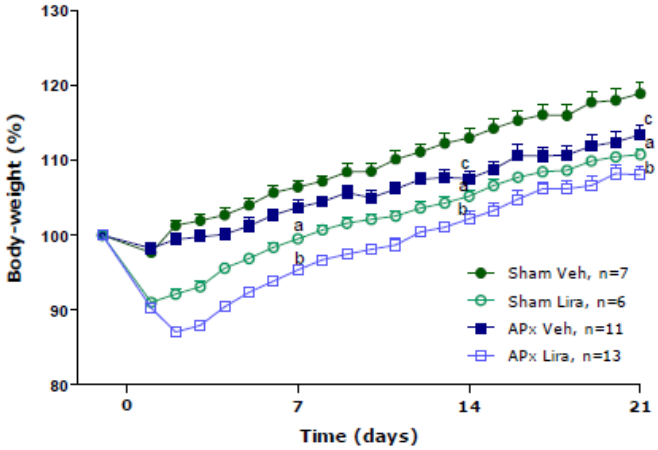


Figure 3 Body weight change in percentage of day -1
Analysis by two-way ANOVA post hoc Bonfferoni (day 7, 14 and 21)
Data are mean ± SEM.
[a]p<0.05 Sham Lira vs. Sham Veh
[b]p<0.05 APx Lira vs. APx Veh
[c]p<0.05 APx Veh vs. Sham Veh

[P17]

AP-ablation lower food intake in rats and liraglutide transiently lowered food consumption in both sham operated and AP-ablated rats (Figure 4, below). In vehicle treated groups, food intake was lower in AP-ablated rats compared to sham operated rats on day -1 and 14. Compared to vehicle treated groups, food intake was decreased during the first 5 days of treatment in both sham-operated and AP-ablated rats, but food consumption was similar between the respective liraglutide and vehicle treated groups from day 6 to the end of treatment.

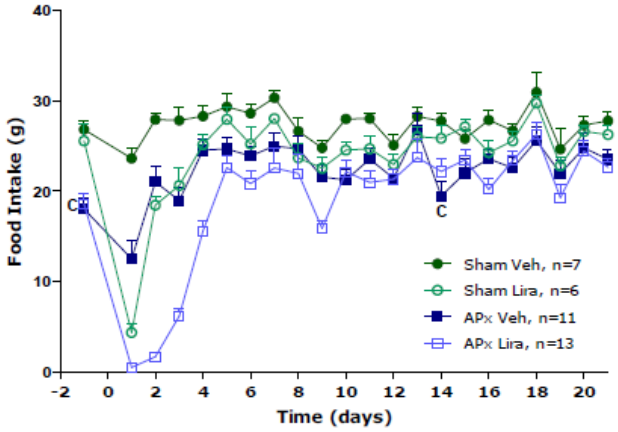


Figure 4 Food intake
Analysis by two-way ANOVA post hoc Bonfferoni (day -1, 7, 14 and 21).
Data are mean ± SEM.
[c]p<0.05 APx Veh vs. Sham Veh

[P18]

AP-ablation had no effect on water intake, but liraglutide transiently decreased water intake in both sham operated and AP-ablated rats (Figure 5, below). Compared to vehicle groups, liraglutide transitently decreased water intake in sham operated rats (day 1, compared to vehicle treated sham operated rats) and in AP-ablated rats (days 1 – 3, compared to vehicle treated AP-ablated rats). In vehicle groups, water intake was significantly increased in AP-ablated rats compared to sham operated rats on day 21, but in the absence of a trend of increased water intake in AP-lesioned rats, this increase was considered incidental.

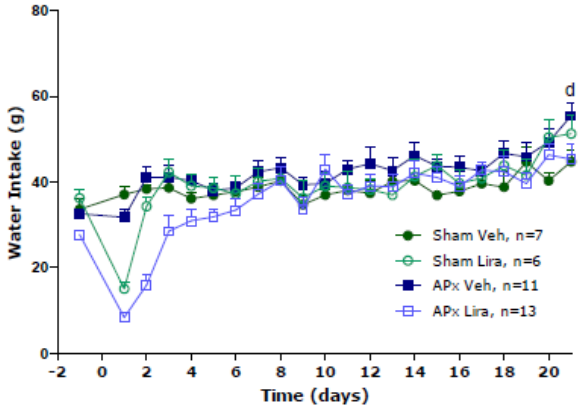


Figure 5 Water intake
Analysis by two-way ANOVA post hoc Bonfferoni (day -1, 7, 14 and 21)
Data are mean ± SEM.
[d]p<0.05 APx Veh vs. APx Lira

[P19]

Study Title: The chronic effect of liraglutide in paraventricular nucleus lesioned animals (report KLYK130303)

Key Study Findings

- Paraventricular nucleus lesions increased food consumption and body weight gain in rats.
- The effects of liraglutide to slow gastric emptying, reduce food consumption, reduce body weight gain, and reduce body weight were not substantially mediated by the paraventricular nucleus.
- The effect of liraglutide to reduce food consumption was transient in sham-operated rats (days 2 and 3 only), but liraglutide reduced food consumption of PVN-lesioned rats during most of the treatment period (days 2, 3, 5, 6, and 8 – 13).

Summary

The effects of liraglutide on body weight and food consumption were evaluated in sham-operated and paraventricular nucleus (PVN)- lesioned male Sprague Dawley rats. Treatment groups are summarized in Table 1, below. Anesthetized rats underwent an operation to lesion the PVN by placing an electrode into the area (bilaterally) and running a 10 μA current for 5 minutes (each side) through it. For the sham operation, electrodes were placed, but a current was not applied. PVN-lesioned and sham-operated rats were allowed to recover for at least 3 days prior to beginning treatment. The presence and absence of PVN lesions were confirmed by microscopic examination of brain tissues slices from all rats on study. Sham-operated rats (groups 1 and 2) and PVN-lesioned rats (groups 3 and 4) were subcutaneously injected with vehicle (0.1% bovine serum albumin in phosphate buffered saline, groups 1 and 3) or 0.2 mg/kg/day liraglutide (groups 2 and 4) twice a day for 14 days.

Table 1 Treatment groups, dose, dose volume and group size

Group	Treatment	Dose	Dose volume, s.c.	Group size, initial	Group size, final only PVN verified**
1) Sham	Vehicle	-	1 ml/kg	n=7	n=7
2) Sham	Liraglutide*	200 μg/kg BID[x]	1 ml/kg	n=8	n=8
3) PVN	Vehicle	-	1 ml/kg	n=16	n=7
4) PVN	Liraglutide*	200 μg/kg BID	1 ml/kg	n=17	n=6

* For test of gastric emptying day 0, liraglutide was dosed s.c. 100 μg/kg (1 ml/kg)
** Only these animals are included in the present report and in raw data tables (appendix 8, and appendix 9)
[x] BID: Twice daily

[P10]

An acute gastric emptying study was performed on the first day of dosing. For this study, rats were administered a single subcutaenous dose of vehicle or 0.1 mg/kg liraglutide followed by a single oral dose of 160 mg/kg acetaminophen and blood levels of acetaminophen were evaluated for up to 180 minutes after acetaminophen dosing. Liraglutide reduced acetaminophen $AUC_{0\text{-}180min}$ indicating liraglutide slowed gastric emptying in both sham-operated and PVN-lesioned rats (Figure 8, below).

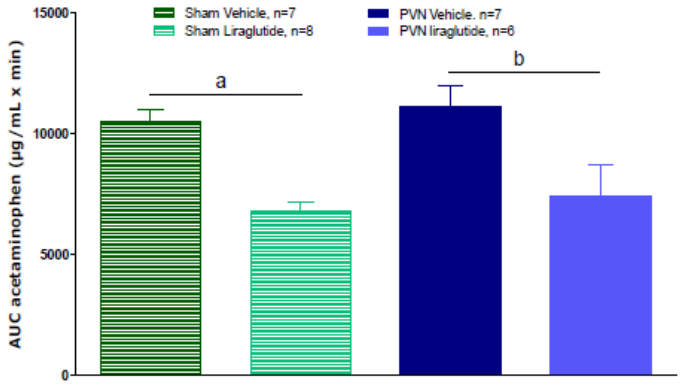


Figure 8 AUC of acetaminophen plasma concentration
Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Liraglutide vs. Sham Vehicle
[b]p<0.05 PVN Liraglutide vs. PVN Vehicle

[P19]

PVN lesions increased body weight gain in rats, but did not ameliorate liraglutide-induced decreased body weight gain. After the gastric emptying study, the dose of liraglutide was increased to 0.2 mg/kg/injection liraglutide twice a day. On day -1, body weight of PVN-lesioned rats (group 3) was significantly higher than sham operated rats (group 1) (Table 2, below). All rats gained wieght during the 14 day treatment period (Figure 3 and Table 2, below). In vehicle treated rats, body weight and body weight gain in PVN-lesioned rats was significantly higher than sham-operated. In liraglutide treated rats, body weight was significantly higher in PVN-lesioned rats compared to sham operated rats on days 2, 3, and 5-14. In sham operated rats, liraglutide reduced body weight gain 41.0% compared to vehicle control during the treatment period and body weight was 3.2% lower compared to control on day 14. In PVN-lesionsed rats, body weight gain during the treatment period was 74.0% lower in the liraglutide-treated group compared to the vehicle-treated group and body weight was 11.0% lower compared to the vehicle-treated group (Table 2, below). In liraglutide treated groups, body weight change as a percentage of the initial body weight was similar in sham operated and PVN-lesioned groups (Figure 4, below).

Table 2 Body weight (start-end) and delta body weight

	Day -1	Day 14	Delta (day -1 to 14)
Sham Veh (n=7)	321.8 ± 17.7	358.4 ± 16.4	36.6 ± 7.8
Sham Lira (n=8)	325.2 ± 15.3	346.8 ± 13.2	21.6 ± 13.7
PVN Veh (n=7)	342.1 ± 11.8 **c**	400.9 ± 19.1 **c**	58.8 ± 13.1 **c**
PVN Lira (n=6)	341.7 ± 13.9	357.0 ± 25.2 **b**	15.3 ± 13.3 **b**

Analysis by one-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[b]p<0.05 PVN Lira vs. PVN Veh
[c]p<0.05 PVN Veh vs. Sham Veh

[P15]

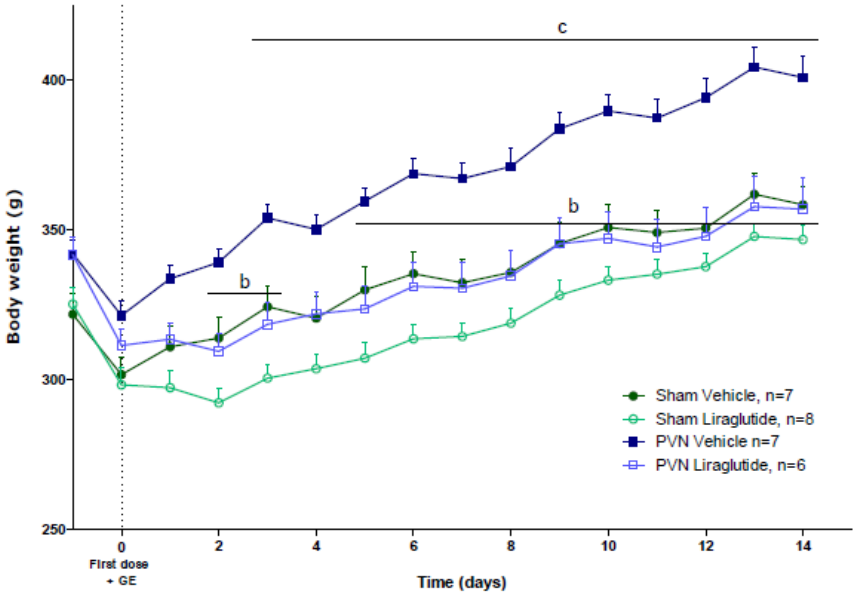


Figure 3 Body weight
Analysis by two-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[b]p<0.05 PVN Liraglutide vs. PVN Vehicle (day 2, 3 and 5-14)
[c]p<0.05 PVN Vehicle vs. Sham Vehicle (day 3-14)

[P14]

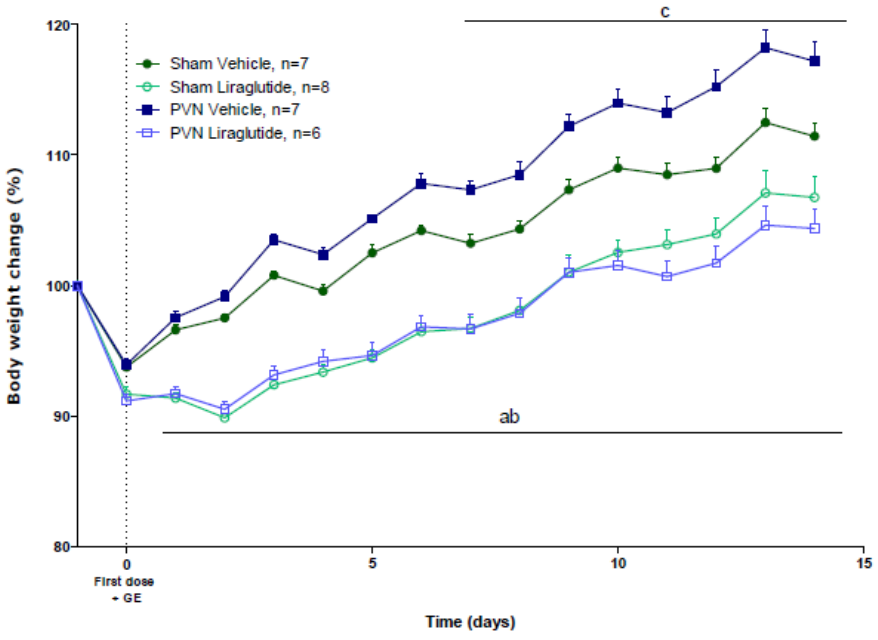


Figure 4 Body weight change in percentage of day -1
Analysis by two-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Liraglutide vs. Sham Vehicle (day 1-14)
[b]p<0.05 PVN Liraglutide vs. PVN Vehicle (day 1-14)
[c]p<0.05 PVN Vehicle vs. Sham Vehicle (day 7-14)

[P16]

In vehicle treated groups, food intake was trended higher in PVN-lesioned rats compared to sham operated rats (Figure 5, below) and the difference reached statistical significance on days 9 and 12. Compared to vehicle-treated rats, liraglutide reduced food consumption in sham-operated rats (days 2 and 3) and in PVN-lesioned rats (days 2, 3, 5, 6, and 8-13). Liraglutide had no significant effect on water intake.

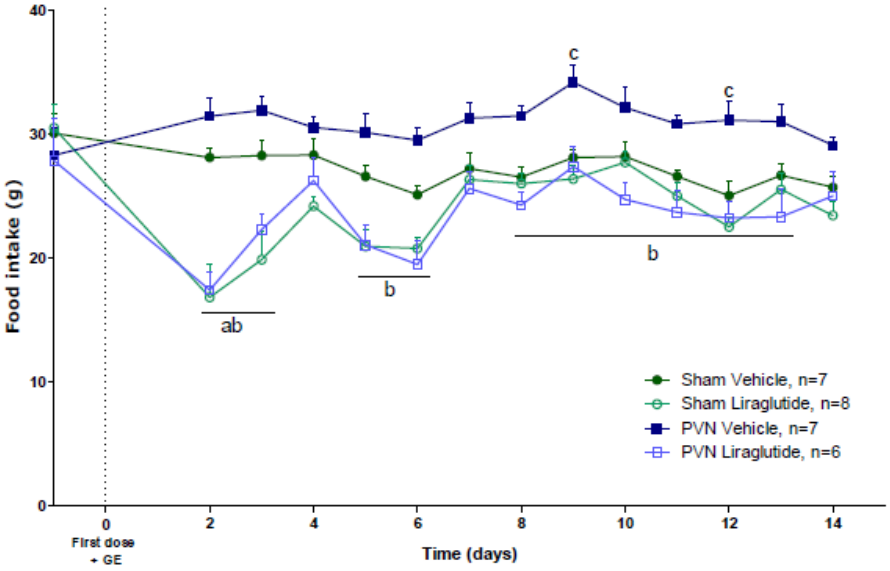


Figure 5 Food intake
Analysis by two-way ANOVA and post hoc Bonferroni. Data are mean ± SEM.
[a]p<0.05 Sham Liraglutide vs. Sham Vehicle (day 2 and 3)
[b]p<0.05 PVN Liraglutide vs. PVN Vehicle (day 2, 3, 5, 6 and 8-13)
[c]p<0.05 PVN Vehicle vs. Sham Vehicle (day 9 and 12)

[P17]

Study Title: The central effects of chronic liraglutide treatment in HE fed DIO rats (report KLYK130304)

Key Study Findings

- Liraglutide reduced body weight of diet-induced obese male rats. In liraglutide-treated DIO rats:
  - decreased body weight was primarily due to reduced food consumption, not increased energy expenditure.
    - both liraglutide treatment food restriction downregulated preproglucagon mRNA in the nucleus of the solitary tract.
    - mRNAs encoding the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) were increased in arcuate nucleus and paraventricular nucleus, without increasing the orexigenic peptides NPY or AgRP
    - food restriction upregulated mRNAs encoding the arcuate hunger signals NPY and AgRP while liraglutide-induced reduced food consumption did not.
  - decreased body weight was primarily due to decreased fat mass.
- Liraglutide significantly increased water consumption.

Summary

Male Sprague Dawley rats (8 months old, 10/group) with obesity induced by a high energy diet (45% fat for 7 months, ad libitum) were subcutaneously injected with 0 (vehicle, phosphate buffered saline) or liraglutide twice a day for 28 days (groups A and B, respectively). The dose in the liraglutide group was escalated from 0.025 liraglutide twice a day on the first day to 0.05 mg/kg/injection twice a day on the second day to 0.1 mg/kg/injection twice a day from day 3 onward. A second vehicle-treated group, group C, was food restricted to match the body weight of the liraglutide-treated group (60% to 90% of average food intake of the liraglutide-treated group). The study design is summarized in Table 1.

Table 1 Test article, dose, concentration, dose volume and group size

Group no.	Test article	Dose (µg/kg)	Concentration (µg/ml)	Dose volume (ml/kg)	Number of rats
A	Vehicle	0	0	0.5	10
B	Liraglutide	100	200	0.5	10
C	Weight matched	0	0	0.5	10

Vehicle: PBS (#BE 17-512F, Biowhitaker)

[SDN 4 Report KLYK130304 P11]

Study parameters were food intake, water intake, body weight, energy expenditure (by indirect calorimetry, 8 / group), and body composition (by magnetic resonance imaging (MRI)) during the treatment period, and after termination at the end of treatment, fat deposits (mesenteric, retroperitoneal, epididymal, and inguinal) were weighed and expression of genes in areas of the brain involved in regulation of appetite and body weight (forebrain including the nucleus accumbens, hypothalamus, and hindbrain including the dorsal vagal complex) were assessed by *in situ* hybridization. *In situ* hybridization in paraformaldehyde-fixed brain tissue sections was performed using ^{33}P-labeled cDNA riboprobes against 12 different mRNAs.

Compared to vehicle control (group A), body weight was significantly lower in diet-induced obese (DIO) rats treated with 0.2 mg/kg/day liraglutide (group B) throughout the treatment period including days 7, 13, 21, and 27 (Figure 1, below). Body weight in the 0.2 mg/kg/day liraglutide group (group B) and weight matched control (group C) was similar during the treatment period, except body weight of the liraglutide group was lower on day 7 due to the initial slower weight loss in the weight matched control group.

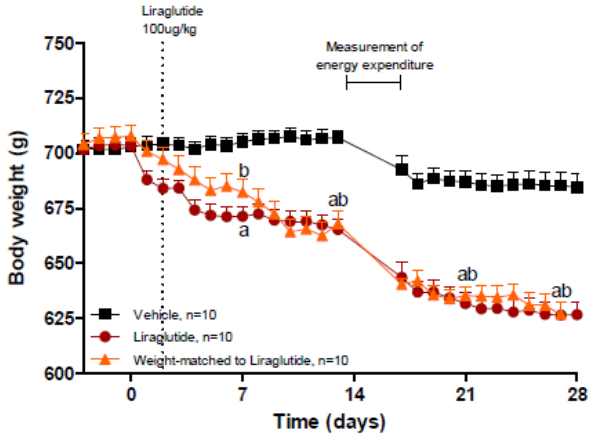


Figure 1 Body weight
a) p<0.05 liraglutide vs. vehicle; b) p<0.05 weight matched vs. vehicle. Statistical analysis performed day -1, 7, 13, 21 and 27. Data are mean ± SEM.

[P15]

Compared to the vehicle control group, food consumption was significnatly decreased in the 0.2 mg/kg/day liraglutide group during the treatment period on days 7, 12, 21, and 26 (Figure 3, below). To achieve a body weight similar to the 0.2 mg/kg/day liraglutide group (group B) during the treatment period, the amount of food fed to the weight matched control group (group C) was significantly lower than the amount fed to the liraglutide group on days 7, 12, and 26. In the 0.2 mg/kg/day liraglutide group (group B), water consumption was significantly increased compared to the vehicle control group (group A) and weight matched group (group C) on days 7, 12, and 24 (Figure 4).

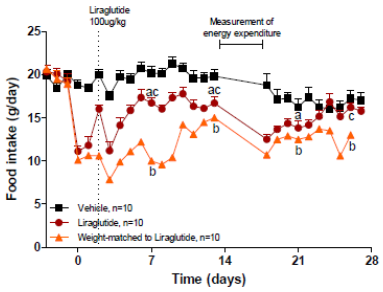


Figure 3 Food intake
a) p<0.05 liraglutide vs. vehicle; b) p<0.05 weight matched vs. vehicle; c) liraglutide vs. weight matched. Statistical analysis performed day 7, 12, 21 and 26 Data are mean ± SEM.

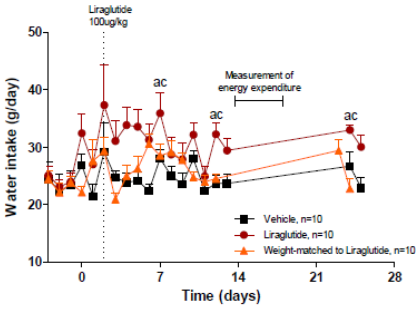


Figure 4 Water intake
a) p<0.05 liraglutide vs. vehicle; c) liraglutide vs. weight matched. Statistical analysis performed day 7, 12, and 24 Data are mean ± SEM.

[P17]

Total body fat determined by magnetic resonsnce imaging (MRI) on study days 14 – 16 showed body fat mass in the liraglutide group (group B) was significantly reduced compared to vehicle control (group A), and weight matched control group (group C) (Figure 5A, below). Lean body mass and water content were were higher in the weight matched control group (group C) compared to the liraglutide group (group B) (Figures 5B and 5C, below).

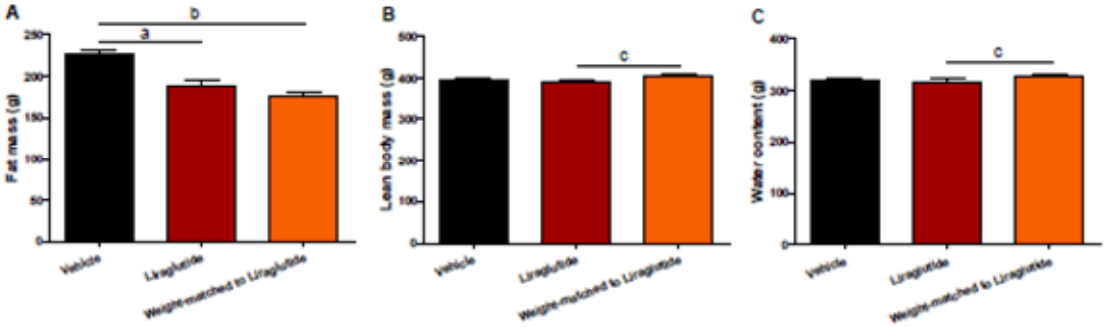


Figure 5 Body composition obtained by MRI scanning day 14-16. A) Fat mass B) lean body mass C) water content
a) p<0.05 liraglutide vs. vehicle; b) p<0.05 weight matched vs. vehicle; c) liraglutide vs. weight matched. Data are mean ± SEM, n=10.

[P18]

Figures 6 and 7 (below) show the mass of mesenteric, peritoneal, subcutaneous inguinal, and epididymal fat was reduced in the 0.2 mg/kg/day liraglutide group (group B) and weight matched control group (group C) compared to vehicle control (group A).

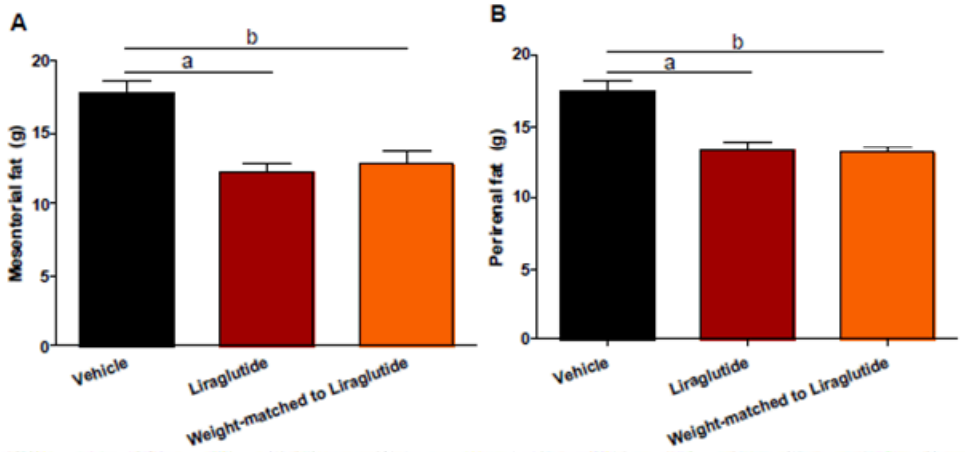


Figure 6 Manually weighing of A) mesenterial and B) perirenal (unilateral) fat depots after 28 days of treatment.
a) $p<0.05$ liraglutide vs. vehicle; b) $p<0.05$ weight matched vs. vehicle. Data are mean ± SEM, n=10.

[P19]

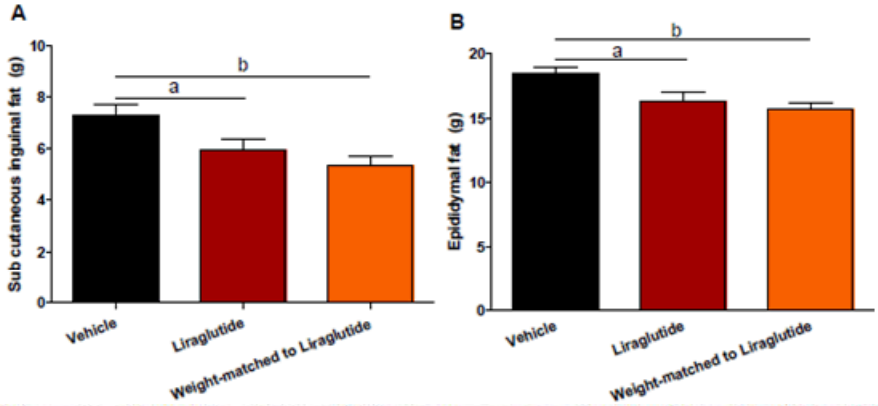


Figure 7 Manually weighing of A) subcutaneous inguinal (unilateral) and B) epididymal (bilateral) fat depots after 28 days of treatment.
a) $p<0.05$ liraglutide vs. vehicle; b) $p<0.05$ weight matched vs. vehicle. Data are mean ± SEM, n=10.

[P20]

Liraglutide had no statistically significant effect on the respiratory quotient (RQ, dCO_2/dO_2) or oxygen consumption (Figures 8 and 9, below), but there was a trend of a decrease in RQ (indicates a higher proportion of fat oxidation compared to carbohydrate oxidation) and increase in oxygen consumption (VO_2) during the dark phase.

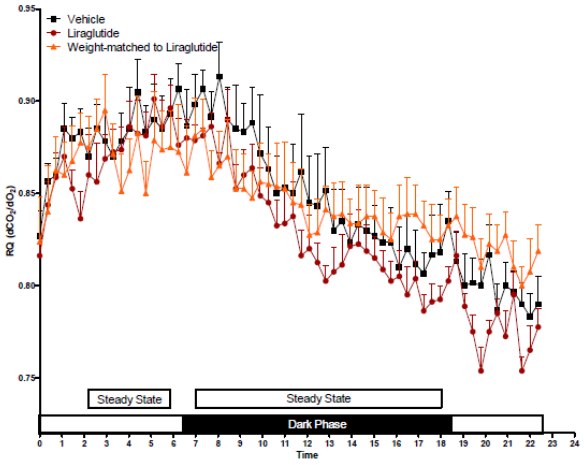


Figure 8 Respiratory quotient (RQ, dCO_2/dO_2) obtained by energy expenditure measurement over a 22.4 hour period.
Measurement was performed in turns over 3 days (Vehicle and Liraglutide: day 15-17, weight matched: day 14-16) on 8 animals per day. Data are mean ± SEM, n=8 (except vehicle: n=6).

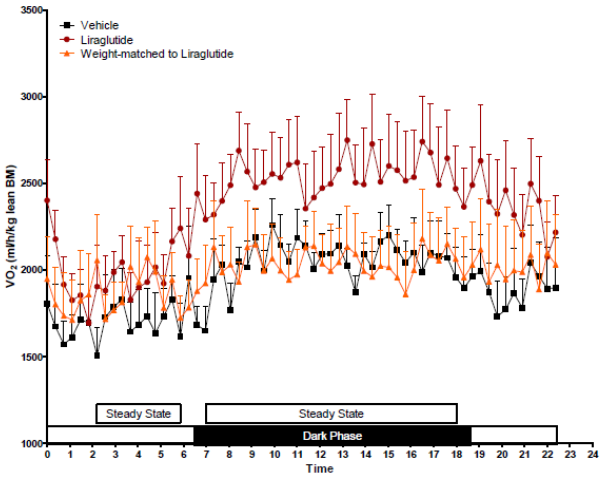


Figure 9 Oxygen consumption (VO_2) obtained by energy expenditure measurement over a 22.4 hour period.
Measurement was performed in turns over 3 days (Vehicle and Liraglutide: day 15-17, weight matched: day 14-16) on 8 animals per day. Data are mean ± SEM, n=8 (except vehicle: n=6).

[P21, 22]

There were substantial differences between the effects of liraglutide and food restriction on transcript levels of genes regulating body weight and appetite in brain. Changes in mRNA levels (compared to vehicle control group) in specific brain regions from DIO rats treated with liraglutide or food restricted weight matched controls are summarized in Table 2, below. Liraglutide increased expression of mRNAs encoding CART (arcuate nucleus and paraventricular nucleus), GLP-1R (area postrema), and thyroid releasing hormone (paraventricular nucleus) and decreased mRNA encoding preproglucagon (solitary tract nucleus). While food restriction in weight matched control group rats also showed a decrease in preproglucagon mRNA in the solitary tract nucleus, it also increased mRNA encoding AgRP, NPY, growth hormone secreting hormone, and leptin receptor in the arcuate nucleus.

Table 2 Change in mRNA relative to vehicle (vehicle mean = 1.00)

Gene	Nucleus	Liraglutide	Weight matched
AgRP	ARC	0.92 ± 0.48	1.74 ± 0.28 bc
NPY	ARC	1.05 ± 0.42	1.41 ± 0.22 bc
POMC	ARC	0.91 ± 0.40	0.98 ± 0.30
CART	ARC	1.61 ±0.27 ac	1.09 ± 0.26
	PVN	1.90 ± 0.41 ac	1.16 ±0.38
	NTS	1.01 ± 0.31	0.88 ± 0.36
	AP	1.08 ± 0.27	1.01 ± 0.22
GLP-1R	ARC	0.77 ± 0.36	0.84 ± 0.32
	PVN	1.15 ± 0.34	1.33 ± 0.33
	AP	1.32 ± 0.18 a	1.12 ± 0.36
GHSR	ARC	0.83 ± 0.27	1.56 ± 0.42 bc
	VMH	1.01 ± 0.22	1.00 ± 0.33
LepR	ARC	1.02 ± 0.45	1.99 ±0.56 bc
	DMH	1.10 ± 0.65	1.61 ± 0.59
rGLUC	NTS	0.64 ± 0.41 a	0.72 ± 0.25 b
TRH	PVN	1.44 ± 0.33 c	0.95 ± 0.49

a) $p<0.05$ liraglutide vs. vehicle; b) $p<0.05$ weight matched vs. vehicle; c) liraglutide vs. weight matched. Data are mean ± SEM, n=10 (some data points are missing due to the brain area not being well represented in some individuals, see Table 22 for details). Relative changes are calculated from gene expression analyses of Agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), Glucagon-like peptide-1 receptor (GLP-1R), growth hormone secretagogue receptor (GHSR), Leptin receptor (LepR), Neuropeptide Y (NPY), Proopiomelanocortin (POMC), preproglucagon (rGLUC) and thyrotropin-releasing hormone (TRH) from hypothalamic (ARC, PVN, DMH, VMH) and brain stem areas (AP and NTS).

[P25]

4.2 Secondary Pharmacology

Study title: In vitro pharmacology: Functional assays - study of liraglutide (report 15582)

In vitro pharmacology screening showed 10 μM liraglutide (batch TQ50297, 6 mg/mL (1.6 mM) stock) was devoid of agonist or antagonist activity at the following human receptors: GLP-2, secretin, GHRH, or VPAC2 (VIP2).

4.3 Safety Pharmacology

Safety pharmacology studies of liraglutide were reviewed under Victoza NDA 22341.

5 Pharmacokinetics/ADME/Toxicokinetics

Brief Summary

Pharmacokinetics, Absorption, Distribution, Metabolism, and Excretion Studies Reviewed Under Victoza NDA 22341

Nonclinical pharmacokinetics, absorption, distribution, metabolism, and excretion studies of liraglutide to support the use of liraglutide for the treatment of type 2 diabetes were reviewed under Victoza NDA 22341.

Liraglutide was formulated in a buffered solution for subcutaneous or intravenous (iv) injection. Plasma pharmacokinetics were determined after single subcutaneous injections (mice, rats, rabbits, monkeys, pigs, and humans) and single iv injections (monkeys, pigs, and humans). Plasma toxicokinetics were determined in single dose studies in mice and rats and multiple dose studies in mice, rats (including pregnant rats), unmated and mated female rabbits, pigs, and monkeys. Plasma liraglutide concentrations were measured using a radioimmunoassay (RIA) that cross-reacted with endogenous GLP-1 (plasma from pigs and rabbits), and later during development, using an enzyme-linked immunosorbent assay (ELISA) that was more 'liraglutide-selective' (plasma from mice, rats, monkeys, and humans).

Bioavailability after subcutaneous injection was 53% in monkeys, 76% in pigs, and 55% in humans. Pharmacokinetics after subcutaneous dosing were linear with dose-proportional increases in peak (Cmax) and total (AUC) exposure in nonclinical species including mice, rats, rabbits, pigs, and monkeys. There were no substantive sex differences in exposure in any species. The plasma elimination half-life was shorter in rats (3.6 hours) than in mice, rabbits, or monkeys (6.7 – 7.1 hours), or pigs and humans (14 - 15 hours). Although total 24 hour systemic exposure ($AUC_{0\text{-}24h}$) was generally higher after repeat dosing, plasma accumulation was minimal in nonclinical species. Consistent with a longer elimination half-life, some accumulation occurred in pigs (1.3-fold) in humans (1.4 – 1.5 fold). Tmax occurred 3 – 11 hours after subcutaneous dosing. Lower protein binding in rats compared to other species may account for the shorter plasma elimination half-life, at least in part. Liraglutide has a shorter half-life after iv dosing compared to subcutaneous dosing, and this is consistent with delayed absorption from subcutaneous injection sites contributing to its persistence in systemic circulation. The apparent volume of distribution (Vz 0.05 – 0.23 L/kg) after iv dosing was consistent with limited extravascular distribution of a highly plasma protein bound drug. Comparison of liraglutide exposure (AUC) versus body weight-based dose (mg/kg) after repeat dosing in different species showed liraglutide exposure was highly correlated with dose across species. Comparison of liraglutide plasma clearance (CL/f) versus body weight (kg) was consistent with allometric scaling with clearance being proportional to body weight across species.

ADAs in blood samples were detected using a RIA precipitating protein-bound radioactivity from plasma incubated with ^{125}I-liraglutide with a limit of sensitivity of >1 µg/mL ADA at plasma liraglutide concentrations $\leq$20 nM. An assay to determine neutralizing activity of ADAs was not developed. ADAs were not detected in repeat subcutaneous dose studies in mice or rats, but they did occur in cynomolgus monkeys treated with 5 mg/kg/day liraglutide for 52 weeks and at 0.25 and 5 mg/kg/day liraglutide in monkeys treated for up to 87 weeks. In repeat subcutaneous dose studies $\geq$52 weeks long, 39% of monkeys in the 5 mg/kg/day liraglutide groups and 6% of monkeys in 0.25 mg/kg/day group developed ADAs

Tissue distribution in albino rats and pigmented rats was determined by whole body autoradiography or scintillation / gamma counting tissue radioactivity after single subcutaneous or iv dosing with 1 of 4 liraglutide radionuclides labeling the drug on the K34R hGLP-1 peptide (^{125}I- or ^{3}H-tyrosine), the glutamate linker (^{14}C-glutamate), or the lipid (^{3}H-palmitate) (Figure 2, below).

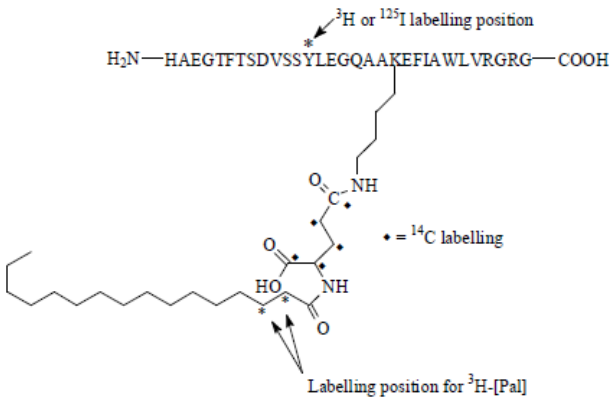


Figure 2 Chemical structure of radio-labeled liraglutide

[SDN 1 module 2.6.1 Nonclinical Written and Tabulated Summaries Introduction P16]

Tissue distribution was determined in male and female albino rats, male pigmented rats, pregnant albino Sprague Dawley rats, and pregnant New Zealand White rabbits. Single subcutaneous dose tissue distribution studies in albino rats using liraglutide radiolabeled on the peptide, linker, or lipid substructures had similar tissue distributions at early time points (<4 hours, with higher amounts in highly vascularized organs including lungs, liver, kidneys, and adrenals), but distribution differed at later time points (>4 hours, higher amounts of radioactivity occurred at the injection site and in liver, kidney, brown fat, and adrenals), probably due to differences in the metabolic fate of different parts of liraglutide (amino acid, linker, or lipid). Plasma levels of radioactivity peaked 4 hours after dosing and liraglutide levels in brain were low suggesting it does not readily cross the blood-brain barrier. Thyroid is a target organ of toxicity in mice and rats, but a microhistoautoradiography study of thyroid and pancreas from rats with samples taken up to 4 hours after administering a single iv dose of [^{3}H-tyr]liraglutide showed radioactivity occurred in pancreatic islets and blood vessel endothelium in thyroid and pancreas, but not in thyroid C-cells. Liraglutide did not bind to melanin-containing organs in rats. Five-day repeat dose tissue distribution studies of liraglutide in pregnant New Zealand White rabbits and albino Sprague Dawley rats showed liraglutide crossed the placenta into fetuses with excretion into amniotic fluid where drug levels were ~ 1% of maternal plasma levels. In rabbits, fetal plasma liraglutide levels were 1.5 – 4.2 % of maternal plasma levels. Radiolabeled liraglutide was secreted in milk of lactating rats, primarily as the parent drug. Based on secretion of liraglutide-related radioactivity in milk, the applicant estimates a pup would consume 0.3 – 3% of the total daily liraglutide dose administered to the dam. Liraglutide in rat milk was largely the intact drug, and its concentration in milk was ~50% of the maternal plasma concentration, but since liraglutide is a lipidated peptide, it probably has low oral bioavailability.

In vitro protein binding studies using equilibrium dialysis showed liraglutide was highly bound to plasma proteins in CD-1 mice (99.5%), NZW rabbits (99.8%), cynomolgus monkeys (99.5%), and humans (99.0%) with slightly lower plasma protein binding in rats (97.0% in Sprague Dawley, 98.9% in ZDF). Liraglutide was highly bound to human serum albumin (99.4%) and human alpha-1 glycoprotein (99.3%). In human plasma, the concentration of liraglutide binding plasma proteins, albumin and alpha-1 glycoprotein (500 – 700 μM and 12 – 31 μM, respectively), are much higher than the Cmax of liraglutide (≤0.12 μM), so it is unlikely liraglutide would alter binding of other highly protein bound drugs. Liraglutide probably binds to fatty acid binding sites on plasma proteins which are not typical binding sites for other highly protein-bound drugs, therefore the risk of pharmacokinetic interactions due to displacement of plasma protein bound drugs is expected to be low. *In vitro*, liraglutide does not alter plasma protein binding of fatty acids (myristic acid or palmitic acid) or drugs (pioglitazone, rosiglitazone,

warfarin, furosemide, tolbutamide, diazepam, glibenclamide, nicardipine, repaglinide, acetylsalicylic acid, valproic acid, metformin, acenocoumarol or phenprocoumon).

In vivo and *in vitro* metabolism studies show liraglutide circulates primarily as the intact parent drug that is metabolized by NEP and DPP-4 with further extensive metabolism of constituent amino acids and palmitic acid. No major human metabolites were identified, but 2 minor metabolites occurred (RtR 1.05 and RtR 1.15) occurred in humans administered a single subcutaneous dose of lipid-labeled [^{3}H-pal]liraglutide. In animals administered [^{3}H-pal]liraglutide, RtR 1.05 was also found in plasma from mice, rats, and monkeys and RtR 1.15 occurred in plasma from rats. *In vitro*, albumin increased the resistance of liraglutide to DPP-4 and NEP, with a much larger effect on NEP-mediated metabolism. *In vitro* metabolism studies using hepatocytes from mice, rats, monkeys, and humans, kidney and liver slices from rats, perfused liver and kidney from rats, and plasma from mice, rats, monkeys, and humans were consistent with NEP and DPP-4 mediated metabolism.

In vitro, liraglutide did not inhibit drug-metabolizing human cytochrome P 450 enzymes (CYPs) CYPs 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Four weeks of treatment with 1 mg/kg/day liraglutide decreased CYP2A1 activity (hepatic steroid hydroxylase, testosterone 7 alpha-hydroxylase) ~2-fold in liver of male rats, but didn't significantly affect liver CYPs 1A2, 2B1/2, 2C11, 2E1, 3A1/2 and 4A.

Excretion of radiolabeled liraglutide was evaluated in rats, monkeys, and humans with recovery of excreted radioactivity and routes of excretion depending on the radiolabeled site. *In vivo* metabolism and excretion studies indicate liraglutide was not excreted intact, its metabolism and excretion were not dependent on kidney or liver, and it was extensively metabolized to amino acids and lipids with further metabolism to water and CO_2. Only 18.9 – 31.2% of total administered radioactivity from a single subcutaneous dose of lipid-labeled [^{3}H-pal]liraglutide in cynomolgus monkeys (0.05 or 5 mg/kg) or humans (0.75 mg) was recovered with 13.5 – 21.7% in urine and 5.4 – 8.8% in feces. The applicant believes radioactivity from[^{3}H-pal]liraglutide was fully metabolized to ^3H_2O and excreted in urine, sweat, and expired air. In rats administered 1 or 7 daily subcutaneous doses of 1 mg/kg ^{14}C-liraglutide labeled in the glutamate linker, 91.7 – 93.2% of the administered radioactivity was recovered with 71.3 – 72.2% in expired air (CO_2), 8.0 – 8.7% in feces, 4.4 – 6.9% in urine, and 8.1% in the carcass. In rats administered 1 or 7 daily subcutaneous doses of 1 mg/kg ^{125}I-liraglutide or a single subcutaneous dose of 1 mg/kg ^{125}I-liraglutide (labeled on tyrosine-19 of K34R hGLP-1(7-37) in liraglutide), 93.9 – 100.6% of the administered radioactivity was recovered with 77.5 – 89.4% in urine, 3.1 – 7.4% in feces, and 7.0 - 10% in the carcass. ^{125}I was primarily excreted in urine as free ^{125}I not associated with protein.

Pharmacokinetics, Absorption, Distribution, Metabolism, and Excretion Studies Reported After Approval of Victoza NDA 22341

After the approval of NDA 22341 in January 2010, plasma liraglutide toxicokinetics were determined in 4 nonclinical studies (see Table 3, below). Reports for studies 210145 (104 week study in CD-1 mice with 26 week administration), LoSi100801 (13 week - Effect in exocrine pancreas from diabetic and non-diabetic ZDF rats) and 209306 (13 week – Effect on C-cell proliferation, rearranged-during-transfection (RET) proto-oncogene activation, and GLP-1R density) were previously submitted to NDA 22341 to fulfill post-marketing requirements PMR 1583-3, PMR 1583-4, and PMR 1583-5, respectively, and these studies were reviewed under NDA 22341. (b) (4)

Table 3 Overview of the non-clinical studies with bioanalysis of liraglutide after submission of the approved Victoza® NDA 22-341

Animal	Study Id.	Study Duration or Type of Study	Bioassay
Mouse			
	209306 [a)]	13 week - Effect on C-cell proliferation, and rearranged-during-transfection (RET) proto-oncogene activation and GLP-1R receptor density	ELISA[b)]
	210145 [a)]	104 week study in CD-1 Mice with 26 Week subcutaneous administration	ELISA[b)]
Rat			(b) (4)
	LoSi100801 [a]	13 week – Effect in exocrine pancreas from diabetic and non-diabetic ZDF rats	ELISA [d]

[a)] Bioanalysis only performed to verify exposure to liraglutide in study.

[b)] The study was analyzed using an updated ELISA method employing Mouse EDTA matrix for preparation of calibration standards (see 4.2.2.1, 210282)

(b) (4)

[d)] The study was analyzed using an ELISA method validated for ZDF rat plasma (see 4.2.2.1, 211027)

[SD1Pharmacokinetics Written Summary P13]

Reports from 5 analytical method validation studies were submitted to NDA 206321. These analytical methods studies validated or qualified an ELISA for determining the concentration of liraglutide in plasma from mice (report 210282) or ZDF rats (report 211027), an ELISA method for determining the concentration of liraglutide in rat plasma using a standard curve prepared in rat plasma (report 211283), and an immunoradiometric assay (IRMA) for determining the concentration of calcitonin in mouse plasma using a mouse calcitonin standard (report 210285) and evaluated the stability of liraglutide in rat plasma stored at -20C (report 211334).

Distribution of subcutaneously injected fluorescent-labeled liraglutide in brain of mice or [^{3}H-hexadecanoyl]liraglutide in brain of mice and rats was evaluated. After 4 days of treatment with fluorescent liraglutide in mice, GLP-1R independent distribution of fluorescent liraglutide occurred in the choroid plexus and zona externa of the median eminence while GLP-1R dependent distribution of liraglutide occurred in other circumventricular regions (area postrema, zona interna of the median eminence, subfornical organ, and organum vasculosum of the lamina terminus) and areas of the hypothalamus (paraventricular, arcuate, dorsomedial hypothalamic, and supraoptic nuclei). After a single subcutaneous dose of [^{3}H-hexadecanoyl]liraglutide in mice or rats, radiolabeled liraglutide-related material occurred in the arcuate nucleus and median eminence in mice and rats) and in the hypothalamic paraventricular nucleus in rats and area postrema in mice. Capillary depletion of brain homogenate from mice administered a single subcutaneous dose of [^{3}H-hexadecanoyl]liraglutide in mice showed radioactivity was located in the brain parenchyma indicating liraglutide-related radioactivity crossed the blood-brain barrier.

5.1 PK/ADME

Analytical Methods

Study title: Validation of NNC 0090-0000-1170 in mouse plasma using Watson LIMS (report 210282)

Summary

A previously validated direct enzyme-linked immunosorbent assay (ELISA) for determining the concentration of liraglutide in EDTA-treated plasma from mice (study 2000-6) was validated to use new computer software to calculate liraglutide concentrations (Watson LIMS instead of Multicalc).

Study title: Validation of an IRMA for determination of calcitonin in mouse EDTA plasma (report 210285)

Summary

A previously validated immunoradiometric assay (IRMA) for determining the concentration of mouse calcitonin in EDTA-treated plasma from mice using rat calcitonin in assay buffer as reference standards (study 205089, 12 pg/mL lower limit of quantitation) was modified to use mouse calcitonin in EDTA-treated plasma from mice as a reference standard. In this mouse calcitonin assay, an anti-rat calcitonin monoclonal antibody immobilized onto plastic beads captures calcitonin from mouse plasma, an ^{125}I-labeled anti-rat calcitonin polyclonal antibody labels it, and radioactivity bound to the washed beads is measured with a gamma counter. The amount of bead-bound radioactivity was directly proportional to the concentration of calcitonin in mouse plasma and calcitonin in mouse plasma was quantified using a standard curve of radioactivity (CPM) versus calcitonin concentration in mouse plasma constructed from reference standards. The lower limit of quantitation was 15 pg/mL.

Study title: Qualification of NNC 0090-1170 in ZDF rat K2EDTA plasma (report 211027, non-GLP)

Summary

Liraglutide (NNC 0090-1170) in EDTA-treated plasma from Zucker Diabetic Fatty (ZDF) rats was quantified by a validated sandwich ELISA. Samples containing liraglutide were incubated for 4 hours at 37C to remove endogenous GLP-1. The ELISA uses antibodies produced at Novo Nordisk. (b) (4) is quantified by light absorbance at λ 450 nm with the amount of absorbance being proportional to the amount of liraglutide in the samples. The concentration of liraglutide in plasma from ZDF rats was determined by comparing absorbance from a plasma sample to a reference curve (λ 450 nm absorbance versus liraglutide concentration).

Study title: Validation of an ELISA for determination of NNC 0090-0000-1170 in rat EDTA plasma (report 211283, non-GLP)

Summary

A previously validated direct enzyme-linked immunosorbent assay (ELISA) for determining the concentration of liraglutide in EDTA-treated plasma from rats using a calibration curve prepared in citrated human plasma (studies 2000-7 and 207095) was validated using a standard curve prepared in EDTA-treated rat plasma.

Study title: Long-term stability of NNC 0090-0000-1170 in rat EDTA plasma (report 211334, non-GLP)

Summary

The long-term stability of liraglutide (NNC 0090-0000-1170) in EDTA-treated rat plasma stored at -20C for up to 6 months was evaluated. Liraglutide concentrations in EDTA-treated rat plasma spiked with known concentrations of liraglutide and stored at -20C for up to 6 months were determined by an ELISA. The applicant concludes liraglutide in EDTA-treated rat plasma stored at -20C was stable for up to 6 months.

5.2 Toxicokinetics

Tabulated summary of plasma liraglutide toxicokinetics in mice, rats, rabbits, and monkeys.

Species	Liraglutide Dose (mg/kg)	Sampe Time	Treatment Duration	Study #	Sex	Liraglutide Plasma Toxicokinetic Parameters		
						Cmax (nM)	AUC (nM.hr)	Tmax (hr)
CD-1 mice	0.03	day 1	single dose	205106	M, F	17	206	6.0
		week 26	104 weeks	204229	M, F	17	128	9.0
		week 52	104 weeks	204229	M, F	7	91	9.0
		week 104	104 weeks	204229	M, F	20	185	6.0
	0.06	day 1	3 days	205050	M	43	576	3.0
		day 3	3 days	205050	M	51	580	6.0
	0.1	day 1	28 days	203261	M, F	75	1073	6.0
		day 28	28 days	203261	M, F	66	812	6.0
	0.2	day 1	single dose	205106	M, F	136	1451	4.5
			13 weeks	204082	M, F	160	1,932	5.0
		week 13	13 weeks	204082	M, F	196	1,959	6.0
		week 26	104 weeks	204229	M, F	125	1,661	5.0
		week 52	104 weeks	204229	M, F	60	723	6.0
		week 104	104 weeks	204229	M, F	141	1,501	8.0
	0.25	day 1	3 days	205050	M	156	2920	6.0
		day 3	3 days	205050	M	268	3040	3.0
	0.5	day 1	28 days	203261	M, F	595	5,889	5.0
		day 28	28 days	203261	M, F	359	4,436	8.0
	1	day 1	single dose	205106	M, F	656	7,756	4.5
			28 days	203261	M, F	1,189	13,950	4.0
			13 weeks	204082	M, F	996	12,424	4.0
		day 28	28 days	203261	M, F	930	9,634	7.0
		week 13	13 weeks	204082	M, F	1,166	15,223	3.5
		week 26	104 weeks	204229	M, F	882	9,232	5.0
		week 52	104 weeks	204229	M, F	1,638	14,355	6.0
		week 104	104 weeks	204229	M, F	1,093	8,153	5.0
	3	day 1	single dose	205106	M, F	2,012	23,545	4.5
		week 26	104 weeks	204229	M, F	2,113	25,385	9.0
		week 52	104 weeks	204229	M, F	2,167	28,805	8.0
		week 104	104 weeks	204229	M, F	3,523	36,830	6.0
	5	day 1	28 days	203261	M, F	5,826	82,252	5.0
			13 weeks	204082	M, F	5,899	74,051	6.0
		day 28	28 days	203261	M, F	5,026	57,609	4.0
		week 13	13 weeks	204082	M, F	6,108	68,534	4.0
NMRI mice	0.02	day 1	single dose	990267	M	10	ND	8.0
	0.2	day 1	single dose	990267	M	58	ND	8.0
	2	day1	single dose	990267	M	592	ND	8.0
ob/ob mice	0.2	day 1	single dose	200031	F	181	2,132	6.0
db/db mice	0.2	day 1	single dose	200031	F	227	2,428	4.0

Species	Liraglutide Dose (mg/kg)	Sampe Time	Treatment Duration	Study #	Sex	Liraglutide Plasma Toxicokinetic Parameters		
						Cmax (nM)	AUC (nM.hr)	Tmax (hr)
SD rats	0.00188	day 1	single dose	970355	F	0.5	4	4.0
	0.02	day 1	single dose	990268	M	6.0	ND	8.0
	0.075	day 1	104 weeks	200240	M, F	32	323	6.0
		week 53	104 weeks	200240	M, F	33	422	8.0
		week 104	104 weeks	200240	M, F	31	423	8.0
	0.1	day 1	28 days	980183	M, F	38	482	6.0
			13 weeks	980189	M, F	69	810	5.0
			26 weeks	200239	M, F	77	872	6.0
		day 28	28 days	980183	M, F	38	505	6.0
		week 13	13 weeks	980189	M, F	46	754	3.0
		week 26	26 weeks	200239	M, F	35	481	7.0
	0.125	day 1	7 days	980180	M, F	85	1,185	6.0
		day 7	7 days	980180	M, F	92	1,020	6.0
	0.2	day 1	single dose	990268	M	74	ND	4.0
	0.25	day 1	7 days	980180	M, F	220	2,567	6.0
			28 days	980183	M, F	115	1,813	8.0
			13 weeks	980189	M, F	267	2,652	7.0
			26 weeks	200239	M, F	188	2,130	5.0
			104 weeks	200240	M, F	112	1,280	8.0
		day 7	7 days	980180	M, F	208	2,603	4.0
		day 28	28 days	980183	M, F	164	2,200	8.0
		week 13	13 weeks	980189	M, F	256	3,088	6.0
		week 26	26 weeks	200239	M, F	107	1,585	9.0
		week 53	104 weeks	200240	M, F	129	1,800	8.0
		week 104	104 weeks	200240	M, F	151	1,785	8.0
	0.75	day 1	104 weeks	200240	M, F	350	5,030	7.0
		week 53	104 weeks	200240	M, F	370	5,895	6.0
		week 104	104 weeks	200240	M, F	398	6,225	8.0
	1	day 1	7 days	980180	M, F	597	7,750	5.0
			28 days	980183	M, F	804	8,198	4.0
			4 weeks (grp 2)	205092	M, F	365	4,927	7.0
			4 weeks (grp 3)	205092	M, F	344	4,939	8.0
			13 weeks	980189	M, F	1,040	12,742	4.0
			26 weeks	200239	M, F	618	8,375	7.0
		day 7	7 days	980180	M, F	448	5,959	5.0
		day 28	28 days	980183	M, F	577	9,074	10.0
		day 30	4 weeks (grp 2)	205092	M, F	463	5,100	6.0
		day 30	4 weeks (grp 3)	205092	M, F	397	4,784	7.0
		week 13	13 weeks	980189	M, F	563	10,698	9.0
		week 26	26 weeks	200239	M, F	568	6,240	7.0
	2	day 1	single dose	990268	M	1,045	ND	4.0
SD rats (unmated and mated)	0.1	day 1 (unmated)	17 days	980186	F	60	680	8.0
		GD 17	17 days	980186	F	75	691	4.0
	0.25	day 1 (unmated)	17 days	980186	F	158	1,980	8.0
		GD 17	17 days	980186	F	214	2,693	8.0
	1	day 1 (unmated)	17 days	980186	F	612	9,148	8.0
		GD 17	17 days	980186	F	1,241	9,211	4.0
[redacted]								(b) (4)

Species	Liraglutide Dose (mg/kg)	Sampe Time	Treatment Duration	Study #	Sex	Liraglutide Plasma Toxicokinetic Parameters		
						Cmax (nM)	AUC (nM.hr)	Tmax (hr)
NZW rabbits (unmated and mated)	0.01	day 1 (unmated)	13 days	980187/8	F	10	140	6.0
		GD 6	13 days	980187/8	F	9	125	6.0
		GD 16	13 days	980187/8	F	11	148	6.0
	0.02	day 1 (unmated)	13 days	980187/8	F	17	245	8.0
	0.03	GD 6	13 days	980187/8	F	19	288	6.0
		GD 16	13 days	980187/8	F	17	280	5.0
	0.1	GD 6	13 days	980187/8	F	36	571	9.0
		GD 16	13 days	980187/8	F	51	766	13.0

Species	Liraglutide Dose (mg/kg)	Sampe Time	Treatment Duration	Study #	Sex	Liraglutide Plasma Toxicokinetic Parameters		
						Cmax (nM)	AUC (nM.hr)	Tmax (hr)
Cynomolgus monkeys	0.05	day 1	28 days	980184	M, F	15	234	11.5
			52 weeks	200241	M, F	30	484	7.3
		day 28	28 days	980184	M, F	13	202	8.5
		week 52	52 weeks	200241	M, F	46	817	8.0
	0.1	day 3	3 days	970455	M, F	185	2,627	4.0
	0.25	week 72	87 weeks	203262	M, F	342	5,352	8.0
		week 87	87 weeks	203262	M, F	423	7,163	8.0
	0.5	day 1	28 days	980184	M, F	187	2,684	8.0
			52 weeks	200241	M, F	417	7,210	7.3
		day 28	28 days	980184	M, F	142	1,858	6.5
		week 52	52 weeks	200241	M, F	423	7,020	7.3
	2.5	day 3	3 days	970455	M, F	1,899	30,933	7.0
	4	day 1	14 days	980181	M, F	3,331	56,425	7.5
		day 14	14 days	980181	M, F	4,923	61,242	6.5
	5	day 1	single dose	980182	M, F	4,489	83,153	8.0
			28 days	980184	M, F	2,098	32,430	9.5
			52 weeks	200241	M, F	5,030	102,900	8.5
		day 3	3 days	970455	M, F	3,725	69,158	8.0
		day 28	28 days	980184	M, F	2,486	25,160	7.0
		week 52	52 weeks	200241	M, F	3,525	59,200	5.2
		week 72	87 weeks	203262	M, F	2,942	51,100	6.0
		week 87	87 weeks	203262	M, F	3,314	52,120	3.8

6 General Toxicology

Brief Summary

Single subcutaneous and iv dose toxicity studies of liraglutide in CD-1 mice and Sprague Dawley rats and repeat subcutaneous dose toxicity studies of liraglutide up to 13 weeks in CD-1 mice, up to 26 weeks in Sprague Dawley rats, and up to 52 weeks in cynomolgus monkeys were reviewed under Victoza NDA 22341 (see table below). No new general single or repeat dose toxicity studies were performed to support the development or marketing of liraglutide for a weight management indication.

Study type and duration	Route of administration	Species
Single-dose toxicity	s.c., i.v.	Mouse and rat
Repeat-dose toxicity		
1 month	s.c.	Mouse, rat and monkey
3 months	s.c.	Mouse, rat and monkey
6 months	s.c	Rat
12 months	s.c.	Monkey

[SD1 Toxicology Written Summary P10]

In general toxicity studies, liraglutide was formulated in a phosphate-buffered aqueous solution that included the excipients phenol (b) (4) and propylene glycol or mannitol (b) (4) Liraglutide was administered by subcutaneous injection, the clinical route of administration. Liraglutide was pharmacologically active in mice, rats, pigs, and cynomolgus monkeys. In general, liraglutide exposure increased with dose with no substantive sex differences in exposure in any species. ADAs didn't occur in mice or rats, but did occur in monkeys in 52- and 87-week repeat dose studies.

Multiples of human exposure for findings in toxicity studies were calculated based on a maximum recommended human dose (MRHD) of 3.0 mg/day liraglutide for a weight management indication yielding steady state pharmacokinetic parameters of Cmax 39 nM and AUC_{0-24h} of 854 nM*h in obese humans, based on population pharmacokinetic analysis. Unless otherwise stated, human exposure multiples were calculated as the ratio of plasma liraglutide AUC_{0-24h} in animals divided by AUC_{0-24h} in obese humans at the MRHD.

A dose-limiting toxicity was not identified in repeat subcutaneous dose toxicity studies of up to 5 mg/kg/day liraglutide in mice or monkeys (up to 80-times and up to 69-times human exposure in mice and monkeys, respectively), but in rats, the maximum tolerated dose (MTD) in repeat dose studies was 1 mg/kg/day liraglutide (approximately 9-times human exposure) based on clinical signs of toxicity (piloerection, rolling / high stepping gait, hunched posture, dark extremities, and thin appearance), inappetence and decreased body weight, and moribund condition leading to euthanasia at 2 and 10 mg/kg/day in a 7-day repeat dose study. Thyroid was the only target organ identified in repeat dose toxicity studies, and only in mice. In thyroid of mice, liraglutide caused ultimobranchial cysts and C-cell focal hyperplasia in 4 and 13 week studies. Carcinogenicity and mechanistic studies of liraglutide, along with data from other GLP-1R agonists approved or in development indicate drug-induced proliferative C-cell lesions (C-cell focal hyperplasia and tumors in mice and rats) are a pharmacological class effect. Although liraglutide caused focal C-cell hyperplasia and tumors in a rat carcinogenicity study, it had no effect on C-cells in rats treated up to 26 weeks (approximately 25% of a rat's total lifespan). Liraglutide did not affect thyroid C-cells in monkeys treated for up to 87 weeks, approximately 8% of their total lifespan. Mild anemia characterized by decreased RBC count, hemoglobin, and hematocrit occurred in mice, rats, and monkeys. Although a cause for decreased RBC parameters was not established, it could be due to hemolysis (mice) and/or bone marrow toxicity (rats). In the absence of correlative histopathology, the toxicological significance of organ weight changes in heart (decreased in rats, increased in monkeys), pancreas (increased in monkeys), and male reproductive organs (decreased seminal vesicle, prostate, and epididymis in rats) was uncertain.

CD-1 Mice

In single subcutaneous or iv dose toxicity studies of 0 or 10 mg/kg liraglutide in mice

(5/sex/dose), liraglutide transiently decreased food consumption and body weight with recovery within 3 days of dosing. The maximum tolerated dose was 10 mg/kg liraglutide subcutaneous or iv.

In an exploratory 7-day repeat subcutaneous dose study of 5 mg/kg/day liraglutide in mice (3/sex/dose), the MTD was 5 mg/kg/day. Food consumption, body weight gain, and in some mice, body weight , were transiently decreased.

In a 4-week repeat subcutaneous dose study of 0.1, 0.5, 1, or 5 mg/kg/day liraglutide in mice (10/sex/dose), systemic exposures were 1.0-, 5.2-, 11-, and 67-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD and NOAEL was 5 mg/kg/day liraglutide, the highest dose tested. Transiently decreased food consumption, decreased body weight gain, and diuresis in all liraglutide groups were considered pharmacologic effects. A mild anemia occurred in males at 1 mg/kg/day and in females at $\geq$ 0.1 mg/kg/day. Thyroid C-cell findings characterized as treatment-related focal hyperplasia by the original study pathologist occurred in 1/10 males in the 1 mg/kg group and in 2/10 females in the 5 mg/kg/day group were later reclassified as unilateral focal perithyroidal C-cells unrelated to treatment by a Pathology Working Group examining liraglutide-associated C-cell findings in mice.

In a 13-week repeat subcutaneous dose toxicity study of 0.2, 1, or 5 mg/kg/day liraglutide in mice (10/sex/dose main study), systemic exposures were 2.3-, 18-, and 80-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD was 5 mg/kg/day liraglutide, the highest dose tested. NOAEL was < 0.2 mg/kg/day based on thyroid focal C-cell hyperplasia and ultimobranchial cysts at $\geq$ 0.2 mg/kg/day. Thyroid was the only target organ. A mild anemia occurred in all liraglutide-treated groups. Transiently decreased food consumption at $\geq$ 1 mg/kg/day liraglutide, transiently decreased body weight gain at $\geq$ 0.2 mg/kg/day in males and at 5 mg/kg/day in females, and diuresis on day 1 at 5 mg/kg/day were considered pharmacological effects.

Sprague Dawley Rats

In single subcutaneous or iv dose toxicity studies of 0 or 10 mg/kg liraglutide in rats (5/sex/dose) sacrificed 15 days after dosing, the MTD was 10 mg/kg subcutaneous or iv. Liraglutide transiently decreased food consumption and body weight gain with recovery within 4 days of dosing.

In 7-day repeat subcutaneous dose studies in rats, the MTD was 1 mg/kg/day liraglutide and the NOAEL was 0.25 mg/kg/day. In the first 7-day study (5 rats /sex/dose) using doses of 0, 0.4, 2, or 10 mg/kg/day liraglutide, the MTD was 0.4 mg/kg/day based on moribund sacrifice of rats in 2 and 10 mg/kg/day groups with clinical signs of toxicity (piloerection, rolling / high stepping gait, hunched posture, dark extremities, and thin appearance), decreased appetence and fecal output, and severe body weight loss. In a second 7-day study using doses of 0, 0.125, 0.25, or 1 mg/kg/day in larger rats, the MTD was 1 mg/kg/day. Decreased food consumption, body weight gain and body weight, and decreased fecal output occurred in all dose groups, but with reduced severity at doses $\leq$ 1 mg/kg/day. Clinical signs of toxicity occurred at $\geq$0.25 mg/kg/day liraglutide, but with reduced severity compared to doses $\geq$ 2 mg/kg/day. Relative heart weight (normalized to body weight) decreased up to 11.1% in males and up to 15.7% in females, but the changes were not dose-related.

In a 28-day repeat subcutaneous dose study of 0.1, 0.25, or 1 mg/kg/day liraglutide in rats (10/sex/dose) with a toxicokinetic satellite group, systemic exposures were 0.6-, 2.6-, and 11-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD was 1 mg/kg/day liraglutide. The NOAEL was 0.25 mg/kg/day liraglutide based on clinical signs of toxicity (hunched posture, piloerection, and rolling or high stepping gait) at 1 mg/kg/day that dissipated after the first week of dosing. Liraglutide reduced food consumption, fecal output, and body weight gain during the first week of treatment at $\geq$ 0.25 mg/kg/day in

males and at 1 mg/kg/day in females and decreased body weight persisted to the end of treatment in high dose males. A mild anemia characterized by decreased RBC count, hematocrit, and hemoglobin occurred at all doses in males and females. Group mean creatine phosphokinase (CPK) was significantly elevated at ≥0.25 mg/kg/day liraglutide in males and females. Decreased absolute weight of thyroid up to 31 % and decreased relative weight up to 30% lacked correlative microscopic pathology. Liraglutide did not increase the incidence of micronucleated erythrocytes in peripheral blood or bone marrow from rats treated for 4 weeks and it had no substantive effect on liver CYP450 content.

A 13-week repeat subcutaneous dose study of 0.1, 0.25, or 1 mg/kg/day liraglutide in rats (10/sex/dose) yielded systemic exposures 0.9-, 2.6-, and 11-times exposure in obese humans at the MHRD, respective, based on AUC comparison. The MTD was 1 mg/kg/day liraglutide, the highest dose tested. The NOAEL was 0.25 mg/kg/day liraglutide based on clinical signs of thin appearance (males only) and hunched posture at 1 mg/kg during the first week of treatment and rolling / high stepping gait and piloerection at 1 mg/kg/day that persisted in high dose recovery group males and females. No target organs were identified. Food consumption was transiently decreased at all doses in males and at ≥0.25 mg/kg/day in females. Dose dependent decreased body weight gain and body weight occurred at ≥0.25 mg/kg/day in males, but body weight of females were not affected. During recovery, body weight gain was 132% higher in high dose recovery group males compared to controls.

In a 26-week repeat subcutaneous dose toxicity study of 0.1, 0.25, or 1 mg/kg/day liraglutide in rats (15/sex/dose) yielding systemic exposure multiples of 0.6-, 1.9-, and 7.3-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD was 1 mg/kg/day liraglutide. The NOAEL was 0.25 mg/kg/day liraglutide based on clinical signs of toxicity at 1 mg/kg/day. Liraglutide transiently decreased food consumption for up to 14 days at all doses in males and at ≥0.25 mg/kg/day in females, dose-dependently decreased body weight gain up to 21 % in males at all doses, and decreased body weight up to 15.7% compared to controls in males at all doses. In the exocrine pancreas, the incidence of up to mild acinar cell atrophy was increased at 1 mg/kg in both males and females and the incidence of minimal focal inflammation was increased in high dose females. Liraglutide did not cause cell proliferation in the pancreas or thyroid C-cells assessed by immunohistochemical staining for proliferating cell nuclear antigen.

Cynomolgus Monkeys

In a rising subcutaneous dose tolerability study of 0.1, 0.5, 2.5, and 5 mg/kg/day liraglutide in cynomolgus monkeys (2/sex) treated for 3 days with a 4 day washout period prior to dose escalation, the MTD was 5 mg/kg/day liraglutide, the highest dose tested. Over the 4 week treatment period, body weight loss of 0.1 kg in both females (5 – 5.6% of starting body weight) was considered a pharmacologic effect. Injection site reaction characterized as enlarged lesion and thickened vein occurred in one male.

In a 14-day tolerability study of 4 mg/kg/day liraglutide subcutaneously injected once a day in monkeys (2/sex), the MTD was 4 mg/kg/day, the highest dose tested. Food consumption transiently decreased in the first week of treatment, and monkeys lost 4.5 – 10.5% of their initial body weight by the end of treatment. A mild regenerative anemia was characterized by decreased RBC count, hematocrit, and hemoglobin and increased reticulocytes. CPK increased 4.4 fold in one male. Injection site reactions had clinical signs of thickening from day 4 onward and necropsy findings of subcutaneous reddening.

In a 28 day repeat subcutaneous dose study of 0.05, 0.5, or 5 mg/kg/day liraglutide in monkeys (3/sex/dose) yielding systemic exposures 0.2-, 2.2-, and 30-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD and NOAEL were 5 mg/kg/day, the highest dose tested. Injection site reaction, attributed to the vehicle, was characterized by subcutaneous thickening starting in week 3 with correlative macroscopic

pathology of reddening and microscopic pathology findings of subacute or chronic fasciitis, hemorrhage, and pigmented macrophages. Decreased body weight gain at ≥ 0.5 mg/kg/day liraglutide in males and females and ~10% decreased body weight at ≥ 0.5 mg/kg/day in males and at 5 mg/kg in females had correlative decreased food consumption during the first week. Absolute and relative weight of pancreas increased 30 – 32% compared to control in males at ≥0.05 mg/kg/day, but the increase lacked correlative histopathology.

In a 13 week repeat subcutaneous dose study of 0.05, 0.5, or 5 mg/kg/day liraglutide in monkeys (4/sex/dose), systemic exposure was confirmed on day 1 and weeks 6 and 13, but toxicokinetic parameters were not determined. The MTD was 5 mg/kg/day liraglutide, the highest dose tested. The NOAEL was <0.05 mg/kg/day based on injection site reactions and increased blood eosinophils at 0.05 mg/kg/day in females. In males, increased eosinophils and injection site reaction occurred at 5 mg/kg/day. Injection site reaction was characterized by clinical signs of subcutaneous thickening with macroscopic pathology findings of reddening and thickening and correlative microscopic chronic active fasciitis. Chronic fasciitis had fibrosis and mononuclear cell infiltrate which occurred in all dose groups including controls, but in affected liraglutide treated monkeys, chronic fasciitis was characterized as active with localized edema and multifocal perivascular infiltration of lymphocytes and eosinophils and increased blood eosinophils. Chronic fasciitis and increased blood eosinophils were not fully reversed in high dose recovery females, and in this group, hemorrhage at the injection site occurred at the end of recovery. A mild anemia occurred at 5 mg/kg/day and in high dose males, and it persisted in the recovery period accompanied by a regenerative response. Alkaline phosphatase dose dependently decreased at ≥0.05 mg/kg/day in males and females and the decrease persisted in high dose recovery groups, but the specific isozyme affected was not identified. Decreased body weight gain and lower body weight compared to controls occurred at 5 mg/kg/day in males and at ≥0.5 mg/kg/day in females. Food consumption transiently decreased during the first week of treatment in all dose groups. Decreased food consumption and decreased body weight gain were considered pharmacological effects, and decreased body weight was reversed at the end of recovery. ADAs were not detected in plasma from monkeys treated for 13 weeks.

In a 52-week repeat subcutaneous dose toxicity study of 0.05, 0.5, or 5 mg/kg/day liraglutide in monkeys (4/sex/dose), systemic exposures were 1.0-, 8.2-, and 69-times exposure in obese humans at the MRHD, respectively, based on AUC comparison. The MTD was 5 mg/kg/day liraglutide, the highest dose tested. The NOAEL was < 0.05 mg/kg/day based on injection site reactions in males and females and inflammatory cell infiltrates in kidney (females) or stomach pylorus (males) at ≥0.05 mg/kg. The incidence of thickened injection site with correlative subcutaneous inflammatory cell infiltration increased with liraglutide dose at ≥0.05 mg/kg/day. In the high dose group, subcutaneous sclerosis, foreign material, and foreign body giant cells also occurred at injection sites. Although treatment-related injection site reactions persisted after a 4 week recovery period, the severity was diminished. Dose-dependent increased relative weight of pancreas (normalized to body weight) occurred at ≥0.05 mg/kg/day in males (53 – 111%) and at ≥0.5 mg/kg/day in females (46 – 79%) with correlative increased mass of exocrine cells and ducts at 5 mg/kg/day in females. Relative weight of heart increased 23 – 49% in males at ≥0.05 mg/kg/day liraglutide, but the increase lacked correlative histopathology. Given the magnitude of relative organ weights increases in males, the 6 – 24% lower body weight in liraglutide groups compared to control does not account for the much larger change in organ weights. Relative increased weight of heart and pancreas were diminished in high dose recovery groups further indicating that the changes were treatment-related. Decreased absolute and relative weight of thymus (normalized to body weight) had correlative pathology findings of atrophy at ≥0.5 mg/kg/day in males. Retrospective analysis of thyroid tissue samples showed liraglutide did not cause C-cell proliferation (assessed by immunohistochemical staining for proliferating cell nuclear antigen) and it did not cause diffuse or focal C-cell hypertrophy (measured by quantitative analysis of calcitonin immunoreactive

cells). A reversible mild anemia (decreased RBC count, hemoglobin, and hematocrit) occurred at ≥0.5 mg/kg/day from week 26 to the end of treatment, and a regenerative response occurred in high dose groups in week 52. Increased total bilirubin at ≥0.5 mg/kg/day in males and at 5 mg/kg/day in females suggests anemia may be hemolytic. In week 52, ALP was decreased in all male liraglutide groups and in females at 5 mg/kg/day, but the isozyme decreased was not identified. Decreased body weight gain and body weight compared to controls in males at ≥0.5 mg/kg/day had correlative transiently decreased food consumption, but it only occurred on the first day of treatment. ADAs cross-reacting with GLP-1 were confirmed in 3 high dose group monkeys (2 males and 1 female).

7 Genetic Toxicology

Brief Summary

In vitro and *in vivo* genetic toxicity assessments of liraglutide were reviewed under Victoza NDA 22341. Liraglutide was not genotoxic *in vitro* or *in vivo*. The approved label for Victoza includes the following statement about genetic toxicity of liraglutide:

"**13.1 Carcinogenesis , Mutagenesis , Impairment of Fertility**

> *Liraglutide was negative with and without metabolic activation in the Ames test for mutagenicity and in a human peripheral blood lymphocyte chromosome aberration test for clastogenicity. Liraglutide was negative in repeat-dose in vivo micronucleus tests in rats."*

No additional reports of liraglutide genetic toxicity studies were submitted to Saxenda NDA 206321.

Genetic toxicity of (b) (4) and (b) (4) leachables in the liraglutide drug product believed to arise from (b) (4) was assessed *in silico* and *in vivo*. *In silico* analysis predicts (b) (4) and (b) (4) would be negative in bacterial reverse mutagenicity assays and positive in vitro in a mammalian chromosomal aberrations assay (see Appendices 1 and 2). Subcutaneously injected (b) (4) and (b) (4) were negative in *in vivo* micronucleus assays in mice (see Appendix 3). (b) (4) and (b) (4) are not genotoxic.

7.3 *In Vivo* Clastogenicity Assay in Rodent (Micronucleus Assay)

Study title:	(b) (4) Induction of micronuclei in the bone marrow of treated mice
Study no:	210060
Study report location:	SD1 module 4.3 Literature References
Conducting laboratory and location:	(b) (4)
Date of study initiation:	18 February 2010
GLP compliance:	claimed (UK and OECD)
QA statement:	page 4
Drug, lot #, and % purity:	(b) (4) (b) (4), lot S87737, 99.4% pure by GC (certificate of analysis page 52)

Key Study Findings

- Subcutaneously injected [redacted] was negative in a bone marrow erythrocyte micronucleus assay in mice.
- Although 2/6 mice in the positive control group were non-responsive and there were apparent problems in staining procedures that may have resulted in the misidentification of polychromatic erythrocytes, the assay was considered valid and negative by CDER's PTCC Genetic Toxicology Subcommittee (see Appendix 3).
- [redacted] was detected in plasma from blood samples taken 2.5 hours after dosing of 1/3 mice administered 450 mg/kg/day [redacted] Systemic exposure to [redacted] was not confirmed by blood sampling.
- Study deficiencies:
  - Report 210313 evaluating plasma [redacted] exposure in 0 and 400 mg/kg/day [redacted] groups (non-GLP) was not submitted.
  - The staining method did not adequately differentiate between normochromatic and polychromatic erythrocytes.
  - 2 positive control group mice were considered non-responsive.

Methods

Doses in definitive study:	0 (vehicle), 100, 200, or 400 mg/kg [redacted]
Frequency of dosing:	2 doses 24 hours apart
Route of administration:	subcutaneous injection
Dose volume:	10 mL/kg
Formulation/Vehicle:	solution in polyethylene glycol 400 (PEG400). Formulations stored at room temperature protected from light and used within 2 hours of preparation.
Species/Strain:	Hsd: ICR (CD-1) mice (6 – 7 weeks old, 27 – 36 g body weight)
Number/Sex/Group:	6 males/dose
Satellite groups:	6 males/0 and 400 mg/kg [redacted] groups for blood sampling of isoflurane anesthetized mice by cardiac puncture 2.5 hours after last dose to confirm plasma exposure (Novo Nordisk report 210313)
Basis of dose selection:	Maximum tolerated dose
Negative control:	Vehicle (PEG400) and a second untreated control group
Positive control:	Cyclophosphamide (CPA) in solution in saline (4.0 mg/mL, 10 mg/kg), a single 40 mg/kg administered orally 24 hours prior to sacrifice

Study Validity

Criteria for a valid assay were:

1. the frequency of micronucleated (MN) bone marrow polychromatic erythrocytes (PCEs) in the vehicle control group is comparable to the historical vehicle control data.
2. At least 5 mice from each dose group are available for analysis.
3. the frequency of MN PCEs in the positive control group is statistically significantly increased compared to the concurrent vehicle control.

Criteria for a positive assay for [redacted] were:

1. a statistically significant increase in the frequency of MN PCE occurs at 1 or more dose levels.
2. the incidence and distribution of MN PCE in individual mice exceeds the laboratories historical control data
3. a dose-response trend in the proportion of MN PCEs.

Criteria for a negative assay for (b) (4) was none of the criteria for a positive assay were met.

If at least 1, but not all of the criteria for a positive assay are met, the result may be considered equivocal.

Table 18 Historical vehicle control ranges

Control Type		Individual PCE %	Individual frequency of micronucleated PCE per 2000 (%)	Animals (%) with 0,1 (or more) micronuclei (for 2000 PCE scored)						
				0	1	2	3	4	5	6+
Vehicle	Mean			(b) (4)						
	SD									
	Median									
	Observed range									
	95% confidence interval for group mean of:									
	4 values									
	5 values									
	6 values									
	7 values									
	8 values									
	9 values									
	10 values									
Positive	Mean	(b) (4)								
	SD									
	Median									
	Observed range									
	95% confidence interval for group mean of:									
	4 values									
	5 values									
	6 values									
	7 values									
	8 values									
	9 values									
	10 values									

(1) Calculated from square root transformed data.

Calculated in July 2009 by (b) (4) from studies started between June 2004 and June 2008. Vehicle control statistics based on 402 animals from 47 studies; positive control statistics based on 169 animals from 24 studies.

[SD1 report 210060 P51]

The micronucleus assay met the following criteria for a valid assay because:

1. At least 5 mice from each dose group were available for analysis.

2. The incidence and distribution of MN PCEs in the vehicle (PEG400) control (b) (4) were consistent with the laboratory's historical vehicle control data (b) (4)).

Although the CPA positive control induced a statistically signficant increase in the frequency of MN PCEs (b) (4), 2 mice in the positive control group were considered non-responders (mice 650 and 682). The incidence of MN PCEs in positive control group mouse 650 (b) (4)) was below the minimum of the historical positive control range of (b) (4) in the first of 2 assessments (see Table 17 below) and near the minimum of the historical control group range in the second assessment for mouse 650 (b) (4) and in both assessments for mouse 682 ((b) (4) The slide analyst opined that initial bone marrow slides prepared from mice 650 and 682 were not optimal for analysis due to large areas of clumped cells, and cells in these areas showed evidence of micronuclei. Despite the lack of a robust increase in MN PCEs in mice 650 and 682, positive control data was accepted due to clear positive responses in 4/6 mice in the positive control group (see Appendix 3).

The %PCEs in (b) (4) groups ((b) (4) was similar to the concurrent untreated and vehicle control groups (b) (4) but below the laboratory historical vehicle control group range ((b) (4) Low %PCE values in negative control and (b) (4) groups were attributed to suboptimal staining with "some slides exhibiting patchy differentiation of PCE and NCE [normochromatic erythocyte] subpopulations".

Results

According to the Toxnet record for (b) (4) (b) (4) the LD_{50} of (b) (4) in mice was (b) (4) mg/kg administered intraperitoneally and (b) (4) mg/kg administered orally. In rats, (b) (4) mmol/kg (b) (4) ((b) (4) mg/kg) injected ip (duration of treatment unknown) caused severe renal necrosis and elevated serum BUN.

Dose Range Finding Study

In dose range-finding studies in male and female CD-1 mice of subcutaneously injected vehicle (PEG400) or (b) (4) the MTD was (b) (4) mg/kg/day with no substantive sex differences in toxicity. Severe clinical signs of toxicity, mortality, and hypothermia occurred in both dose group in an initial dose range-finding study of 0 (vehicle, PEG400) or (b) (4) mg/kg (b) (4) in mice (2/sex/control, 3/sex/ (b) (4) dose) using a dose volume of (b) (4) mL/kg with 2/2 male and 2/2 female vehicle treated mice and 3/3 females sacrificed moribund within 1 day after the first dose and 3/3 males in the (b) (4) mg/kg/day (b) (4) group surviving to the end of the study, 2 days after the second daily dose. Due to toxicity of the vehicle, the dose volume was reduced from 20 mL/kg to 10 mL/kg in a second range-finding study of 0 (vehicle), 400, 450, 500, or 650 mg/kg/day (b) (4) in mice (3/sex/dose, 10 mL/kg). The second dose-range finding study established a MTD of (b) (4) mg/kg/day due to severe clinical signs of toxicity at (b) (4) mg/kg/day (b) (4) (unkempt, ptosis, edema in males and females and decreased activity and piloerection in males) and (b) (4) mg/kg/day (unkempt, ptosis, edema, decreased activity in males and unkempt, edema, ptosis, hunched posture, tachypnea, and tremors in females) with mortality occurring in 1/3 males and

1/3 females at (b) (4) mg/kg/day and in 1/3 males and 2/3 females at (b) (4) mg/kg/day. A gelatinous layer beneath the skin was noted in decedents. In the second range finding study, no substantive gender differences were observed for toxicity from (b) (4) and clinical signs of eye closure attributed to (b) (4) and clinical signs of unkempt appearance and slight swelling (edema) attributed to the vehicle were reversed by day 4. There were no clinical signs in mice surviving 2 days after the second dose. (b) (4) decreased body temperature to 31.5C at (b) (4) mg/kg/day and 24.4C at (b) (4) mg/kg/day, and there were no substantive treatment-related changes in body weight.

Group mean bodyweight changes (Day 1 to Day 4) were as follows:

Males at (b) (4) mg/kg/day = +3.0% / Females at (b) (4) mg/kg/day = +3.7%
Males at mg/kg/day = +0.0% / Females at mg/kg/day = +3.6%
Males at mg/kg/day = -2.6% / Females at mg/kg/day = -3.7%.

[SD1 report 210060 P27]

Micronucleus Assay

Dose groups in the micronucleus assay are summarized in Table 2, below.

Table 2 Dose Levels – Micronucleus Experiment

Group Number	Treatment	Dose volume (mL/kg)	Dose (mg/kg/day)	No. of animals	Sample time (hours after administration)
1	Vehicle control [a]	10	0	6M	24
2	Untreated control	-	UTC	6M	24
3	(b) (4)	10	100	6M	24
4		10	200	6M	24
5		10	400	6M	24
6	Positive control [b]	10	40	6M	24

M Male
a PEG 400
b Cyclophosphamide (CPA), given as a single administration on the day prior to bone marrow harvest
UTC Untreated control

[SD1 report 210060 P15]

Study parameters were clinical signs including post-dose observations, body weight (prior to dosing and necropsy), and bone marrow sampling. Twenty-four hours after the last dose, mice were euthanized by an intraperitoneal sodium pentobarbitone overdose and cervical dislocation. Bone marrow was harvested from both femurs of each mouse and washed in fetal calf serum. Bone marrow smears on slides were air dried, fixed in methanol, rinsed in distilled water, stained with diluted Giemsa stain, rinsed, dried, cleared with xylene, dried, and mounted with coverslips. Slides were examined by light microscopy under blinded conditions (slides were labeled with study number, sampling time, sex, date of preparation, and animal number). The relative proportions of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were determined after counting at least 1000 erythrocytes per mouse. Counting continued until at least 2000 PCEs per mouse were examined. All MN PCEs observed during counting were recorded. Erythrocyte parameters were %PCE (to evaluate bone marrow toxicity in individual mice and groups), frequency of MN PCEs (MN per 2000 PCEs) and % MN PCEs (individual mice and group)

Mouse 670 in the (b) (4) mg/kg/day (b) (4) group was sacrificed moribund approximately 1 hour after the first dose with clinical signs of unkempt appearance, edema, decreased activity,

hypothermia, and ptosis, but a cause of death was not established by gross necropsy. Clinical signs of unkempt appearance and edema occurred in mice treated with 0 (vehicle), (b) (4) mg/kg/day (b) (4) for up to 4 hours after dosing with unkempt appearance persisting during the entire 2-day treatment period. At (b) (4) mg/kg/day (b) (4) in mice surviving to scheduled sacrifice, an additional clinical sign of decreased activity was noted 1 to 2 hours after dosing. Compared to the concurrent vehicle control, body temperature was unaffected by treatment with (b) (4) mg/kg/day (b) (4) but body temperature was reduced 2 hours after dosing on day 1 in 2/5 mice in the (b) (4) mg/kg/day. Body weight was unaffected by treatment with (b) (4) A low incidence of mortality and clinical signs confirmed (b) (4) mg/kg/day (b) (4) was an appropriate high dose.

Dosing formulations were acceptable. Analysis of dosing formulations (vehicle, 10, 20, or 40 mg/mL (b) (4) showed (b) (4) was not in the vehicle and the concentration of (b) (4) was +/- 10% of the nominal concentration in all samples. (b) (4) was detected in plasma from 1/3 mice in a satellite group administered (b) (4) mg/kg/day (b) (4)

Systemic exposure to (b) (4) was assessed in plasma from blood samples taken 2.5 hours after subcutaneous dosing with (b) (4) mg/kg (b) (4) Systemic exposure to (b) (4) was not confirmed because it was only detected in plasma from 1/3 mice. The bioanalysis report was not submitted in the final report.

In a bone marrow erythrocyte micronucleus assay of 0 (vehicle, PEG400), 100, 200, or 400 mg/kg (b) (4) subcutaneously injected once a day for 2 consecutive days (24 hours between doses) in male CD-1 mice, bone marrow from femur was sampled 24 hours after the last dose. Clinical signs of unkempt appearance and slight swelling was observed in all mice (vehicle or (b) (4) groups). In the (b) (4) mg/kg (b) (4) group, clinical signs of decreased activity occurred in all mice and 1/6 mice with additional signs of eye closure and hypothermia was sacrificed moribund. Group mean %PCE in (b) (4) groups were below the historical vehicle control range and similar to the concurrent vehicle control. There was no evidence of bone marrow toxicity in any group.

The frequency of MN PCEs in (b) (4) groups were not significantly different from the concurrent vehicle control group (Tables 16 and 17, below). The frequency of MN PCEs in mouse 662 in the (b) (4) mg/kg (b) (4) group was slightly elevated (b) (4), Table 17, below), but consistent with the frequency of MN PCEs in individual mice in the historical vehicle control data (Table 18, above). Up to (b) (4) mg/kg (b) (4) injected subcutaneously once a day for 2 consecutive days was negative in a bone marrow micronucleus assay in mice.

Table 16 (b) (4) **Summary and statistical analysis of micronucleus data**

Treatment (mg/kg/day)	Cell Total	% PCE	MN PCE	% MN PCE	SD	MN PCE / 2000 PCE	Heterogeneity X^2	Heterogeneity S	Contingency X^2C	Contingency S
(b) (4)										

[SD1 report 210060 P49]

Study title: [redacted] Induction of micronuclei in the bone marrow of treated mice

Study no:	210064
Study report location:	SD1 module 4.3 Literature References
Conducting laboratory and location:	[redacted]
Date of study initiation:	18 February 2010
GLP compliance:	claimed (UK and OECD)
QA statement:	page 4
Drug, lot #, and % purity:	[redacted] ([redacted]), lot 1446142, 99.9% pure by GC (certificate of analysis page 50)

Key Study Findings

- Subcutaneously injected [redacted] was negative in a bone marrow erythrocyte micronucleus assay in mice.
- Although there were problems in staining procedures that may have resulted in the misidentification of polychromatic erythrocytes, the assay was considered valid and negative by the PTCC Genetic Toxicology Subcommittee (see Appendix 3).
- [redacted] was detected in plasma from blood samples taken 2.5 hours after dosing of 2/3 mice administered 400 mg/kg/day [redacted] The bioanalysis report was not submitted.
- Study deficiencies:
  - Report 210313 evaluating plasma [redacted] exposure in 0 and 400 mg/kg/day [redacted] groups (non-GLP) was not submitted.
  - The report evaluating [redacted] concentration in dosing formulations was not submitted.
  - The staining method may not have adequately differentiated between normochromatic and polychromatic erythrocytes.

Methods

Doses in definitive study:	0 (vehicle), 100, 200, or 400 mg/kg (b) (4)
Frequency of dosing:	2 doses 24 hours apart
Route of administration:	subcutaneous injection
Dose volume:	10 mL/kg
Formulation/Vehicle:	solution in 10% ethanol in polyethylene glycol 400 (10E/PEG400). Formulations stored at room temperature protected from light and used within 2 hours of preparation.
Species/Strain:	Hsd: ICR (CD-1) mice (6 – 7 weeks old, 28 – 35 g body weight)
Number/Sex/Group:	6 males/dose
Satellite groups:	3 males/0 and 400 mg/kg (b) (4) groups for blood sampling of isoflurane anesthetized mice by cardiac puncture 2.5 hours after last dose to confirm plasma exposure (Novo Nordisk report 210313)
Basis of dose selection:	Maximum tolerated dose
Negative control:	Vehicle (10% ethanol in PEG400 (10E/PEG)) and a second untreated control group
Positive control:	Cyclophosphamide (CPA) in solution in saline (4.0 mg/mL, 10 mg/kg), a single 40 mg/kg administered orally 24 hours prior to sacrifice

Study Validity

Criteria for a valid assay were:

1. the frequency of micronucleated (MN) bone marrow polychromatic erythrocytes (PCEs) in the vehicle control group was comparable to the historical vehicle control data.
2. at least 5 mice from each dose group were available for analysis.
3. the frequency of MN PCEs in the positive control group was statistically significantly increased compared to the concurrent vehicle control.

Criteria for a positive assay for (b) (4) were:

1. a statistically significant increase in the frequency of MN PCEs occurs at 1 or more (b) (4) dose levels.
2. the incidence and distribution of MN PCEs in individual mice in affected (b) (4) groups exceeds the laboratories historical control data
3. a dose-response trend in the proportion of MN PCEs.

Criteria for a negative assay for (b) (4) were none of the criteria for a positive assay were met.

If at least 1, but not all of the criteria for a positive assay are met, the result may be considered equivocal.

Table 18 Historical vehicle control ranges CD1-Mouse - males

Control Type		Individual PCE %	Individual frequency of micronucleated PCE per 2000 (%)	Animals (%) with 0,1 (or more) micronuclei (for 2000 PCE scored)						
				0	1	2	3	4	5	6+
Vehicle	Mean									(b) (4)
	SD									
	Median									
	Observed range									
	95% confidence interval for group mean of:									
	4 values									
	5 values									
	6 values									
	7 values									
	8 values									
	9 values									
	10 values									
Positive	Mean		(b) (4)							
	SD									
	Median									
	Observed range									
	95% confidence interval for group mean of:									
	4 values									
	5 values									
	6 values									
	7 values									
	8 values									
	9 values									
	10 values									

(1) Calculated from square root transformed data.

Calculated in July 2009 by (b) (4) from studies started between June 2004 and June 2008. Vehicle control statistics based on 402 animals from 47 studies; positive control statistics based on 169 animals from 24 studies.

[SD1 report 210064 P49]

The micronucleus assay met the following criteria for a valid assay because:

1. At least 5 mice from each dose group were available for analysis.

2. The incidence and distribution of MN PCEs in the vehicle (10E/PEG400) control ((b) (4) were consistent with the laboratory's historical vehicle control data ((b) (4)).

3. The positive control, CPA, induced a statistically significant increase in the frequency of MN PCEs ((b) (4) compared to control.

The %PCEs in (b) (4) groups (b) (4) at 100, 200, and 400 mg/kg/day (b) (4) respectively) was similar to the concurrent untreated and vehicle control groups (b) (4) respectively), but below the minimum of the laboratory historical vehicle control group range (b) (4) Low %PCE values in negative control and (b) (4) groups were attributed to suboptimal staining with "some slides exhibiting patchy differentiation of PCE and NCE subpopulations".

Results

According to the Toxnet database record for (b) (4) ((b) (4) , the LD_{50} in mice was (b) (4) mg/kg administered intraperitoneally and (b) (4) mg/kg administered orally.

Dose Range Finding Study

In dose range-finding studies in male and female CD-1 mice of vehicle (10E/PEG400) or (b) (4) subcutaneously injected once a day for 2 days and maintained for 2 additional days to evaluate reversibility, the MTD was (b) (4) mg/kg/day with no substantive sex differences in toxicity. Dose groups in the range-finding study are shown in Table 1 (below).

Table 1 Dose Levels – Range-Finder Experiment

Group Number	Treatment	Dose volume (ml/kg)	Dose (mg/kg)	No. of animals
1	(b) (4)	20	400	3M 3F
2	Vehicle[a]	20	-	2M 2F
3	Vehicle[a]	10	-	2M 2F
4	(b) (4)	10	300	3M 3F
5		10	400	3M 3F
6		10	550	3M 3F

M Male
F Female
a 10% Ethanol in PEG400

[SD1 report 210064 P15]

In the first dose-range finding study of 0 (vehicle,10E/PEG400) or (b) (4) mg/kg/day (b) (4) using a dose volume of (b) (4) mL/kg, severe clinical signs of toxicity in the vehicle group (unkempt appearance and edema in males and females with ptosis and decreased activity in males prior to moribund sacrifice within 4 hours after dosing on day 1) and the (b) (4) mg/kg/day (b) (4) group (unkempt appearance with or without ptosis on day 2, with more severe clinical signs of decreased activity edema, hypothermia, hunched posture, lethargy, and bradyapnea in males) and mortality in vehicle control group mice (2/2 males and 2/2 females sacrificed moribund within 4 hours after dosing on day 1) and (b) (4) mg/kg/day group (1/3 females found dead on day 2 and 3/3 males sacrificed moribund within 4 hours after dosing on day 2), toxicity was attributed to the volume of vehicle administered subcutaneously. The dose volume was reduced from 20 mL/kg to 10 mL/kg in a second range-finding study.

Reviewer note: In the reviewer's opinion, clinical signs of unkempt appearance and ptosis in females in the 20 mL/kg vehicle control group were probably not severe enough to warrant early termination (see Table 14 (excerpted), below).

Table 14 (b) (4) Clinical observations – Range-Finder - Females

Group/Treatment (mg/kg/day)	Animal number and sex	Clinical Sign												
		Day 1 (hours after administration)					Day 2 (hours after administration)						Day 3	Day 4
		Imm	0.5	1	2	4	Pre-dose	Imm	0.5	1	2	4		
(b) (4)	82F													(b) (4)
	83F													
	84F													
Vehicle (0) 20 ml/kg	784F													
	785F													
Vehicle (0) 10 ml/kg	391F													
	392F													

[SD1 report 210064 P44]

In dose range-finding studies of vehicle (10E/PEG400) or 300, 400, or 550 mg/kg/day (b) (4) in male and female CD-1 mice (3/sex/dose) subcutaneously injected once a day for 2 consecutive days, the MTD was (b) (4) mg/kg/day due to moribund sacrificed of 3/3 males with clinical signs (unkempt, hypothermia, edema, tachypnea, ptosis, decreased activity, and tremors within 1 hour after the first or second dose) and 2/3 females within clinical signs (unkempt, hypothermia, edema, tachypnea, decreased activity, tremors, ptosis within 1 hour after the first or second dose) in the (b) (4) mg/kg/day (b) (4) group. Four hours after dosing on day 1, core body temperature was dose-dependently decreased 5% and 23% in males and 3% and 14.6% in females at (b) (4) mg/kg/day (b) (4) but not on day 2 (Table 10, below).

Table 10 (b) (4) Body Temperatures, Range-finder Experiment- Males

Group/Treatment (mg/kg/day)	Animal number and sex	Body temperatures					
		Day 1 (hours after administration)			Day 2 (hours after administration)		Day 3
		Pre-dose	2	4	2	4	
(b) (4)	79M						(b) (4)
	80M						
	81M						
Vehicle (0) 20 ml/kg	782M						
	783M						
Vehicle (0) 10 ml/kg	389M						
	390M						
(b) (4)	589M						
	590M						
	591M						
(b) (4)	397M						
	398M						
	399M						
(b) (4)	798M						
	799M						
	800M						

Footnotes on page 46

Table 11 (b) (4) Body Temperatures, Range-finder Experiment- Females

Group/Treatment (mg/kg/day)	Animal number and sex	Body temperatures					
		Day 1 (hours after administration)			Day 2 (hours after administration)		Day 3
		Pre-dose	2	4	2	4	
(b) (4)	82F						(b) (4)
	83F						
	84F						
Vehicle (0) 20 ml/kg	784F						
	785F						
Vehicle (0) 10 ml/kg	391F						
	392F						
(b) (4)	592F						
	593F						
	594F						
(b) (4)	400F						
	898F						
	899F						
(b) (4)	100F						
	74F						
	75F						

[SD1 report 210064 P39,40]

There were no substantive sex differences in toxicity. Unkempt appearance persisted in 1/3 females in the (b) (4) mg/kg/day (b) (4) group surviving for 2 days after the last dose. Clinical signs

in 0 (vehicle), (b) (4) mg/kg/day (b) (4) groups were unkempt appearance and edema lasting for up to 4 hours after dosing with ptosis also occurring in (b) (4) groups on day 1, but not on day 2. Unkempt appearance persisted 1 day after the last dose (day 3) in 0, (b) (4) mg/kg/day (b) (4) and 2 days after the last dose (day 4) in vehicle control groups only. At (b) (4) or (b) (4) mg/kg/day, (b) (4) had no effect on body weight. A gelatinous layer beneath the skin was noted in the majority of decedents.

Micronucleus Assay

In a bone marrow erythrocyte micronucleus assay of 0 (vehicle, 10E/PEG400), 100, 200, or 400 mg/kg (b) (4) subcutaneously injected once a day for 2 consecutive days (24 hours between doses) or a single oral dose of 40 mg/kg CPA in male CD-1 mice (6/group), bone marrow from femur was sampled 24 hours after the last dose. Dose groups in the micronucleus assay are summarized in Table 2, below.

Table 2 Dose Levels – Micronucleus Experiment

Group Number	Treatment	Dose volume (ml/kg)	Dose (mg/kg/day)	No. of animals	Sample time (hours after administration)
1	Vehicle control [a]	10	-	6M	24
2	Untreated	-	-	6M	24
3	(b) (4)	10	100	6M	24
4		10	200	6M	24
5		10	400	6M	24
6	Positive control [b]	10	40	6M	24

M Male
a 10% Ethanol in PEG400
b Cyclophosphamide (CPA) administered once on Day 2

[SD1 report 210064 P15]

Study parameters were clinical signs including post-dose observations, core body temperature, body weight (prior to dosing and necropsy), and bone marrow sampling. Twenty four hours after the last dose, mice were euthanized by an intraperitoneal sodium pentobarbitone overdose and cervical dislocation. Bone marrow was harvested from both femurs of each mouse and washed in fetal calf serum. Bone marrow smears on slides were air dried, fixed in methanol, rinsed in distilled water, stained with diluted Giemsa stain, rinsed, dried, cleared with xylene, dried, and mounted with coverslips. Slides were examined by light microscopy under blinded conditions (slides were labeled with study number, sampling time, sex, date of preparation, and animal number). The relative proportions of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were determined after counting at least 1000 erythrocytes per mouse. Counting continued until at least 2000 PCEs per mouse were examined. All MN PCEs observed during counting were recorded. Erythrocyte parameters were %PCE (to evaluate bone marrow toxicity in individual mice and groups), frequency of MN PCEs (MN per 2000 PCEs) and % MN PCEs (individual mice and group). Because of suboptimal staining characteristics, a second set of slides were stained, but "This resulted in patchy differentiation of the polychromatic erythrocyte and normochromatic erythrocyte subpopulations." A third set of slides was prepared from "bone marrow residues" the day after harvesting bone from mice 748 and 750 in the (b) (4) mg/kg/day (b) (4) group "due to background artefacts present on the initial slides that could be confused with micronuclei" and data from the initial slides from mice 748 and 750 were rejected and not reported.

Dosing formulations were acceptable. Analysis of dosing formulations (vehicle, 10, 20, or 40 mg/mL (b) (4) showed (b) (4) was not in the vehicle and the concentration of (b) (4) was +/-

10% of the nominal concentration in all samples. Systemic exposure to (b) (4) was assessed in plasma from blood samples taken 2.5 hours after subcutaneous dosing with (b) (4) mg/kg (b) (4) Systemic exposure to (b) (4) was detected in plasma from 2/3 mice administered (b) (4) mg/kg/day (b) (4) but plasma (b) (4) levels were not reported and the applicant believes peak plasma exposure occurred prior to the sampling resulting in the absence of (b) (4) in plasma of 1/3 mice in the (b) (4) mg/kg/day (b) (4) satellite group. The bioanalysis report was not submitted in the final report.

There were no unscheduled deaths in any dose group. Clinical signs of unkempt appearance and edema occurred in 0 (vehicle), (b) (4) mg/kg/day (b) (4) groups for up to 4 hours after dosing with unkempt appearance persisting during the entire 2-day treatment period. At (b) (4) mg/kg/day (b) (4) decreased activity, hunched posture, and tremors also occurred within 1 to 4 hours after dosing on days 1 and 2. Body weight was unaffected by treatment with (b) (4) The %PCE was similar in all dose groups, including untreated control and vehicle control groups indicating (b) (4) was not toxic to bone marrow.

The frequency of MN PCEs in (b) (4) groups ((b) (4)) were not significantly different compared to the concurrent vehicle control ((b) (4) (Tables 16, below) and the frequencies of MN PCEs in individual mice in (b) (4) groups ((b) (4)) (Table 17, below) were within the historical vehicle control group range (b) (4)) (Table 18, above). Up to (b) (4) mg/kg (b) (4) injected subcutaneously once a day for 2 consecutive days was negative in a bone marrow micronucleus assay in mice.

Table 16 (b) (4) Summary and statistical analysis of micronucleus data

Treatment (mg/kg/day)	Cell Total	% PCE	MN PCE	MN PCE / 2000 PCE	% MN PCE	Standard Deviation	Heterogeneity X2	Heterogeneity Significance	Contingency X2C	Contingency Significance
(b) (4)										

[SD1 report 210064 P47]

Table 17 (b) (4), Individual animal micronucleus frequencies

Treatment (mg/kg)	Animal Number	PCE Count	NCE Count	% PCE	Total PCE Count	MN PCE	% MN PCE
(b) (4)							

8 Carcinogenicity

Brief Summary

Carcinogenicity

Repeat subcutaneous dose studies evaluating carcinogenicity of liraglutide in mice and rats were reviewed under Victoza NDA 22341. The approved label for Victoza includes the following black box warning primarily based on results from rodent carcinogenicity studies:

"FULL PRESCRIBING INFORMATION

WARNING: RISK OF THYROID C-CELL TUMORS

Liraglutide causes dose-dependent and treatment-duration-dependent thyroid C-cell tumors at clinically relevant exposures in both genders of rats and mice. It is unknown whether Victoza causes thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans, as human relevance could not be ruled out by clinical or nonclinical studies. Victoza is contraindicated in patients with a personal or family history of MTC and in patients with Multiple Endocrine Neoplasia syndrome type 2 (MEN 2). Based on the findings in rodents, monitoring with serum calcitonin or thyroid ultrasound was performed during clinical trials, but this may have increased the number of unnecessary thyroid surgeries. It is unknown whether monitoring with serum calcitonin or thyroid ultrasound will mitigate human risk of thyroid C-cell tumors. Patients should be counseled regarding the risk and symptoms of thyroid tumors [see Contraindications (4), Warnings and Precautions (5.1) and Nonclinical Toxicology (13.1)].”

The approved label for Victoza includes the following statement regarding carcinogenicity of liraglutide in mice:

“**13.1 Carcinogenesis , Mutagenesis , Impairment of Fertility**

A 104-week carcinogenicity study was conducted in male and female CD-1 mice at doses of 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide administered by bolus subcutaneous injection yielding systemic exposures 0.2-, 2-, 10- and 45-times the human exposure, respectively, at the MRHD of 1.8 mg/day based on plasma AUC comparison. A dose-related increase in benign thyroid C-cell adenomas was seen in the 1.0 and the 3.0 mg/kg/day groups with incidences of 13% and 19% in males and 6% and 20% in females, respectively. C-cell adenomas did not occur in control groups or 0.03 and 0.2 mg/kg/day groups. Treatment-related malignant C-cell carcinomas occurred in 3% of females in the 3.0 mg/kg/day group. Thyroid C-cell tumors are rare findings during carcinogenicity testing in mice. A treatment-related increase in fibrosarcomas was seen on the dorsal skin and subcutis, the body surface used for drug injection, in males in the 3 mg/kg/day group. These fibrosarcomas were attributed to the high local concentration of drug near the injection site. The liraglutide concentration in the clinical formulation (6 mg/mL) is 10-times higher than the concentration in the formulation used to administer 3 mg/kg/day liraglutide to mice in the carcinogenicity study (0.6 mg/mL).”

The maximum recommended human dose (MRHD) for the weight management indication is 3.0 mg/day liraglutide, a dose yielding steady state plasma liraglutide $AUC_{0\text{-}24h}$ 854 nM*h in obese adult humans. In the 104-week carcinogenicity study in CD-1 mice, doses of 0.03, 0.2, 1, and 3 mg/kg/day liraglutide administered by bolus subcutaneous injection yielded systemic exposures 0.2-, 2-, 10- and 43-times the exposure in obese humans, respectively, at the MRHD of 3.0 mg/day based on plasma AUC comparison.

The approved label for Victoza includes the following statement regarding carcinogenicity of liraglutide in rats:

“**13.1 Carcinogenesis , Mutagenesis , Impairment of Fertility**

A 104-week carcinogenicity study was conducted in male and female Sprague Dawley rats at doses of 0.075, 0.25 and 0.75 mg/kg/day liraglutide administered by bolus subcutaneous injection with exposures 0.5-, 2- and 8-times the human exposure,

respectively, resulting from the MRHD based on plasma AUC comparison. A treatment-related increase in benign thyroid C-cell adenomas was seen in males in 0.25 and 0.75 mg/kg/day liraglutide groups with incidences of 12%, 16%, 42%, and 46% and in all female liraglutide-treated groups with incidences of 10%, 27%, 33%, and 56% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. A treatment-related increase in malignant thyroid C-cell carcinomas was observed in all male liraglutide-treated groups with incidences of 2%, 8%, 6%, and 14% and in females at 0.25 and 0.75 mg/kg/day with incidences of 0%, 0%, 4%, and 6% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. Thyroid C-cell carcinomas are rare findings during carcinogenicity testing in rats."

In the 104-week carcinogenicity study in Sprague Dawley rats, doses of 0.075, 0.25, and 0.75 mg/kg/day liraglutide administered by bolus subcutaneous injection yielded systemic exposures 0.5-, 2-, and 8-times the exposure in obese humans, respectively, at the MRHD of 3.0 mg/day based on plasma AUC comparison.

Mechanistic toxicity studies evaluating the human relevance of liraglutide-induced rodent thyroid C-cell tumors were reviewed under Victoza NDA 22341, but these mechanistic studies did not support the conclusion that liraglutide-induced thyroid C-cell tumors in mice and rats were not relevant to humans. The approved label for Victoza includes the following statement regarding human relevance of liraglutide-induced rodent thyroid C-cell tumors:

"**13.1 Carcinogenesis , Mutagenesis , Impairment of Fertility**

Human relevance of thyroid C-cell tumors in mice and rats is unknown and could not be determined by clinical studies or nonclinical studies [see Boxed Warning and Warnings and Precautions (5.1)]."

Approval of Victoza NDA 22341 included 2 nonclinical post marketing requirements (PMRs) to further assess the potential human risk of liraglutide-induced proliferative lesions of thyroid C-cells:

1. PMR 1583-3, a 2-year study in mice to determine if 26 weeks of liraglutide treatment increases the lifetime risk of thyroid C-cell tumors.

2. PMR 1583-5, a 13-week mouse study to determine if liraglutide-induced focal C-cell hyperplasia depends on a thyroid glucagon-like peptide-1 (GLP-1) receptor and rearranged-during-transfection (RET) proto-oncogene activation.

PMR 1583-3 was fulfilled by study 210145, a 104-week study in CD-1 mice subcutaneously administered 0 (vehicle), 0.2, 1.0, or 3.0 mg/kg/day liraglutide for 26 weeks followed by a 78 week recovery period. Mice treated with 3.0 mg/kg/day liraglutide for 25% of their total life span (26 weeks) were at an increased risk of developing proliferative thyroid C-cell lesions (preneoplastic focal C-cell hyperplasia in males and benign C-cell adenoma in females) for up to 78 weeks after treatment was stopped. By the end of the 26-week treatment period, liraglutide increased plasma calcitonin up to 14.1-fold in males at $\geq$0.2 mg/kg/day and up to 4.0-fold in females at $\geq$1.0 mg/kg/day and induced thyroid focal C-cell hyperplasia at $\geq$0.2 mg/kg/day in males (4.3% to 22.7% affected) and at 0.2 and 3.0 mg/kg/day in females (8.3% and 31.8%, respectively), but liraglutide did not induce C-cell tumors. By the end of the 78 week recovery period, plasma calcitonin remained elevated 1.4- to 1.8-fold in males previously treated with $\geq$0.2 mg/kg/day liraglutide, a low incidence of thyroid focal C-cell hyperplasia exceeded the incidence in concurrent and historical control groups at 3.0 mg/kg/day liraglutide in males (3.8%

affected at 3.0 mg/kg/day compared to 2.7% in concurrent controls and 0% in historical controls), and benign C-cell adenoma occurred in 1 female in the 3.0 mg/kg/day group (1.3% at 3 mg/kg/day liraglutide compared to 0% in concurrent and historical controls). Due to the low incidence of proliferative C-cell lesions in thyroid in male and female high dose recovery group mice and the unusual finding of a low incidence of proliferative C-cell lesions in concurrent control group male mice, a clear relationship to liraglutide treatment was not established for proliferative C-cell lesions in high dose recovery groups. Whether or not transient exposure to liraglutide increases the lifetime risk of proliferative C-cell lesions in mice could not be adequately addressed by study 210145 because of the uncertainty that proliferative C-cell lesions in high dose recovery groups were related to liraglutide treatment.

PMR 1583-5 was fulfilled by study 209306, a 13-week repeat dose study of wild-type (WT) CD-1 mice subcutaneously injected once a day with 0 (vehicle), 0.03, 0.3, or 3 mg/kg/day liraglutide and GLP-1R-deficient (GLP1rKO) mice subcutaneously injected once a day with 0 or 3 mg/kg/day liraglutide. Calcitonin immunohistochemical analysis of thyroid tissue sections to identify C-cells showed liraglutide increased the incidence of minimal to slight diffuse C-cell hyperplasia at $\geq$ 0.3 mg/kg/day in both male and female WT mice, but 3 mg/kg/day liraglutide did not cause C-cell hyperplasia in GLP1rKO mice. Dose-dependent increased plasma calcitonin at the end of treatment occurring at $\geq$ 0.03 mg/kg/day liraglutide in WT mice (2.8- to 24.2-fold compared to controls in males and 2.5- to 12.1-fold in females) was consistent with drug-related C-cell hyperplasia. In GLP1rKO mice, liraglutide did not increase plasma calcitonin. Immunohistochemical analysis of thyroid tissue sections from WT mice in the control group without C-cell hyperplasia and 3 mg/kg/day liraglutide group with C-cell hyperplasia showed liraglutide induced phosphorylation of serine 235/236 in ribosomal protein S6 in normal and hyperplastic C-cells, but without inducing phosphorylation of tyrosine 1062 in RET or serine 217/S221 in mitogen-activated protein kinase kinase 1 or 2 (MEK1/2). Liraglutide induced thyroid C-cell hyperplasia in mice was GLP-1R-dependent and liraglutide activated ribosomal protein S6 in normal and hyperplastic C-cells in mice, but liraglutide did not activate RET or MEK1/2 in mouse C-cells.

Although there is no evidence from clinical studies that liraglutide causes C-cell tumors in humans, a 13 August 2014 pharmacovigilance review of post-marketing safety information for Victoza authored by Drs. Debra Ryan and Carolyn Tabak in CDER's Division of Pharmacovigilance I identified 9 cases of medullary thyroid cancer (MTC), a rare C-cell cancer in humans, in the FDA Adverse Event Reporting System (FAERS) database. Based on clinical evidence confirming the diagnosis of MTC and a causality assessment performed by the Division of Oncology Products 2, 7/9 of the FAERS case reports were consistent with sporadic MTC and 6 of these MTC cases were possibly related to liraglutide treatment. The possibility that liraglutide was a causal determinant of MTC in 6 cases reported in FAERS could not be excluded. (b) (5)

9 Reproductive and Developmental Toxicology

Brief Summary

Toxicity studies evaluating the effects of subcutaneously injected liraglutide on fertility in rats, embryofetal development in rats and rabbits, and pre- and post-natal development in rats were reviewed under Victoza NDA 22341. (b) (4)

Fertility

The approved label for Victoza includes the following statements regarding the effects of liraglutide on fertility:

*"**13.1 Carcinogenesis , Mutagenesis , Impairment of Fertility***

In rat fertility studies using subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide, males were treated for 4 weeks prior to and throughout mating and females were treated 2 weeks prior to and throughout mating until gestation day 17. No direct adverse effects on male fertility was observed at doses up to 1.0 mg/kg/day, a high dose yielding an estimated systemic exposure 11- times the human exposure at the MRHD, based on plasma AUC. In female rats, an increase in early embryonic deaths occurred at 1.0 mg/kg/day. Reduced body weight gain and food consumption were observed in females at the 1.0 mg/kg/day dose."

In the fertility studies in Sprague Dawley rats, the high dose of 1.0 mg/kg/day liraglutide yielded a systemic exposures 11-times the exposure in obese humans at the MRHD of 3.0 mg/day, based on plasma AUC comparison.

Embryofetal Development

The approved label for Victoza includes the following statements regarding potential effects of liraglutide on embryofetal development based on toxicity studies in rats and rabbits and postnatal development in rats:

*"**8.1 Pregnancy***

Pregnancy Category C.

There are no adequate and well-controlled studies of Victoza in pregnant women. Victoza should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Liraglutide has been shown to be teratogenic in rats at or above 0.8 times the human systemic exposures resulting from the maximum recommended human dose (MRHD) of 1.8 mg/day based on plasma area under the time-concentration curve (AUC). Liraglutide has been shown to cause reduced growth and increased total major abnormalities in rabbits at systemic exposures below human exposure at the MRHD based on plasma AUC.

Female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide beginning 2 weeks before mating through gestation day 17 had estimated systemic exposures 0.8-, 3-, and 11-times the human exposure at the MRHD based on plasma AUC comparison. The number of early embryonic deaths in the 1 mg/kg/day group increased slightly. Fetal abnormalities and variations in kidneys and blood vessels, irregular ossification of the skull, and a more complete state of ossification occurred at all doses. Mottled liver and minimally kinked ribs occurred at the highest dose. The incidence of fetal malformations in liraglutide treated groups exceeding concurrent and historical controls were misshapen oropharynx and/or narrowed opening into larynx at 0.1 mg/kg/day and umbilical hernia at 0.1 and 0.25 mg/kg/day."

In the combined fertility and embryofetal developmental toxicity study in female Sprague Dawley rats, subcutaneous doses of 0.1, 0.25, and 1.0 mg/kg/day liraglutide yielded systemic exposures 0.8-, 3-, and 11-times the exposure in obese humans at the MRHD of 3.0 mg/day liraglutide, based on plasma AUC comparison.

Pregnant rabbits given subcutaneous doses of 0.01, 0.025 and 0.05 mg/kg/day liraglutide from gestation day 6 through day 18 inclusive, had estimated systemic exposures less than the human exposure at the MRHD of 1.8 mg/day at all doses, based on plasma AUC. Liraglutide decreased fetal weight and dose dependently increased the incidence of total major fetal abnormalities at all doses. The incidence of malformations exceeded concurrent and historical controls at 0.01 mg/kg/day (kidneys, scapula), ≥ 0.01 mg/kg/day (eyes, forelimb), 0.025 mg/kg/day (brain, tail and sacral vertebrae, major blood vessels and heart, umbilicus), ≥ 0.025 mg/kg/day (sternum) and at 0.05 mg/kg/day (parietal bones, major blood vessels). Irregular ossification and/or skeletal abnormalities occurred in the skull and jaw, vertebrae and ribs, sternum, pelvis, tail, and scapula; and dose-dependent minor skeletal variations were observed. Visceral abnormalities occurred in blood vessels, lung, liver, and esophagus. Bilobed or bifurcated gallbladder was seen in all treatment groups, but not in the control group."

In an embryofetal developmental toxicity study in pregnant rabbits, subcutaneous doses of 0.01, 0.025, and 0.05 mg/kg/day liraglutide yielded systemic exposures below exposure in obese humans at the MRHD of 3.0 mg/day liraglutide, based on plasma AUC comparison.

"In pregnant female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide from gestation day 6 through weaning or termination of nursing on lactation day 24, estimated systemic exposures were 0.8-, 3-, and 11-times human exposure at the MRHD of 1.8 mg/day, based on plasma AUC. A slight delay in parturition was observed in the majority of treated rats. Group mean body weight of neonatal rats from liraglutide-treated dams was lower than neonatal rats from control group dams. Bloody scabs and agitated behavior occurred in male rats descended from dams treated with 1 mg/kg/day liraglutide. Group mean body weight from birth to postpartum day 14 trended lower in F_2 generation rats descended from liraglutide-treated rats compared to F generation rats descended from controls, but differences did not reach statistical significance for any group."

In the pre- and post-natal development toxicity study in pregnant rats given subcutaneous doses of 0.1, 0.25, or 1.0 mg/kg/day liraglutide, estimated systemic exposures were 0.8-, 3-, and 11-times the exposure in obese humans at the MRHD of 3.0 mg/day liraglutide, based on plasma AUC comparison.

Lactation

The approved label for Victoza includes the following statements regarding potential excretion of liraglutide in milk based on a study in lactating rats:

*"**8.3 Nursing Mothers***

It is not known whether Victoza is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for tumorigenicity shown for liraglutide in animal studies, a decision should be made whether to discontinue nursing or to discontinue Victoza, taking into account the importance of the drug to the mother. In lactating rats, liraglutide was excreted unchanged in milk at concentrations approximately 50% of maternal plasma concentrations."

Juveniles

The approved label for Victoza includes the following statement regarding the use of liraglutide in pediatric patients:

*"**8.4 Pediatric Use***

Safety and effectiveness of Victoza have not been established in pediatric patients. Victoza is not recommended for use in pediatric patients."

(b) (4)

(b) (4)

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(b) (4)

10 Special Toxicology Studies

Local Toxicity

Studies evaluating local toxicity of liraglutide dosing formulations were reviewed under Victoza NDA 22341. In humans, liraglutide (6 mg/mL) is subcutaneously injected once a day. The 0.5 mL dose volume for the only proposed maintenance dose of 3.0 mg/day liraglutide for weight management is 1.7-times higher the 0.3 mL dose volume for the maximum recommended human dose of 1.8 mg/day liraglutide approved for the treatment of type 2 diabetes mellitus. Local toxicity of subcutaneously injected liraglutide was assessed in repeat dose toxicity studies of liraglutide up to 13 weeks in CD-1 mice, up to 26 weeks in Sprague Dawley rats, and up to 52 weeks in cynomolgus monkeys, and in 104-week carcinogenicity studies in CD-1 mice and Sprague Dawley rats, but the concentration of liraglutide in the marketed formulation (6.0 mg/mL) is at least 3-times higher than the concentration of liraglutide used in nonclinical dosing formulations in repeat dose nonclinical studies. Irreversible injection site reactions consisting of inflammation and fibrosis occurred in the 52-week chronic toxicity study in monkeys treated with the high dose of 5 mg/kg/day liraglutide using a liraglutide dosing solution that was 3-fold more dilute than the clinical formulation. In the mouse carcinogenicity study, 3 mg/kg/day liraglutide subcutaneously injected into the dorsal surface caused fibrosarcomas on the dorsal skin and subcutis in males using a dosing formulation with a liraglutide concentration that was 10-fold lower than the concentration of liraglutide in the clinical formulation. Local toxicity studies of liraglutide in pigs using 0.2 mL of dosing formulations similar to or identical to those used in clinical studies of liraglutide evaluating its use for the treatment of type 2 diabetes showed injection site reactions in pigs occurred in vehicle-treated and liraglutide-treated groups, but inflammation was more severe in drug-treated animals.

Two additional local toxicity study reports were submitted to Saxenda NDA 206321; a study evaluating local toxicity of the to-be-marketed formulation in pigs (report 208224) and a study evaluating local toxicity of 6 mg/mL liraglutide injected into New Zealand White rabbits by routes not intended for clinical use (intramuscular, iv, and intra-arterial) (report 208428). Both study reports were submitted in the 2009 annual report for IND 61040 investigating the use of liraglutide for the treatment of type 2 diabetes during review of NDA 22341 for Victoza. In a single dose local toxicity study using the to-be-marketed formulation of 6 mg/mL liraglutide (0.2 mL, 1.2 mg/injection liraglutide, batch TQ50297 manufactured in November 2007) subcutaneously injected into the dorsal surface of LYD pigs, mild injection site reactions consisting of microscopic injection site inflammation, hemorrhage, and/or fibrosis occurring 2 to 5 days after dosing were attributed to the vehicle. In a single dose study using 0.06 mL (0.36 mg) of the to-be-marketed formulation of 6 mg/mL liraglutide administered by single intramuscular, intravenous, or intra-arterial injection in rabbits (approximately 0.09 mg/kg) to determine local toxicity of the drug given by routes not intended for clinical use, only mild injection site reactions occurred 2 or 5 days after dosing.

Human Relevance of Liraglutide-Induced Rodent Thyroid C-cell Tumors

Liraglutide caused thyroid C-cell adenomas (benign) and carcinomas (malignant) in rats and mice and malignant fibrosarcomas in the dorsal skin and subcutis in male mice. Mechanistic studies evaluating the human relevance of liraglutide-induced thyroid C-cell tumors

in mice and rats were reviewed under Victoza NDA 22341. To dismiss human relevance, the applicant proposed a mode-of-action for liraglutide-induced thyroid C-cell tumors in rodents, and then evaluated the mode-of-action in an extensive series of mechanistic studies including *in vivo* studies in mice, rats, and monkeys. The novel mode-of-action for GLP-1R agonist induced thyroid C-cell tumors included the following key steps:

1. GLP-1R agonist activation of thyroid C-cell GLP-1Rs.
2. GLP-1R activation stimulates calcitonin secretion (calcitonin is a 'prehyperplasia' biomarker).
3. Calcitonin synthesis increases.
4. Persistent calcitonin secretion and increased synthesis causes C-cell hyperplasia.
5. C-cell hyperplasia progresses to C-cell tumors, including progression of benign adenomas to carcinomas.

Tables 6 and 12 (below) summarizing species comparison of key events in the mode-of-action for GLP-1R agonist induced C-cell tumors were included in a human relevance assessment document first issued in February 2008 (Victoza NDA 22,41, Table 6) and re-issued in October 2013 (Saxenda NDA 206321, Table 12). Table 6 (February 2008) and Table 12 (October 2013) are essentially the same, except Table 12 includes an additional key event of thyroid C-cell GLP-1 receptor (GLP-1R) density in thyroid tissue, which is noted as high in rats and marginal in humans.

Table 6 Species comparison - key events in GLP-1R agonist induced rodent C-cell proliferation

Key event	Rat	Mouse	Cynomolgus	Man
C-cell GLP-1 receptor presence (density in cell lines)	Yes (high)	Yes	Yes	Yes (low)
GLP-1 receptor activation (magnitude of response)	Yes (high)	-	-	(No) (marginal)
Calcitonin release	Yes	Yes	No	No
↑ calcitonin synthesis	Yes	Yes	-	-
C-cell hyperplasia	Yes	Yes	No	-
C-cell neoplasia	Yes	Yes	-	-

- indicates key events not evaluated in the stated species

[NDA22341 Rodent C-cell findings: Assessment of human relevance Feb 2008 P41]

Table 12 Species comparison - key events in GLP-1R agonist induced rodent C-cell proliferation

Key event	Rat	Mouse	Cynomolgus	Man
C-cell GLP-1 receptor presence (density in cell lines)	Yes (high)	Yes	Yes	Yes [1] (low)
C-cell GLP-1 receptor presence (density in thyroid tissue)	Yes (high)	-	-	No/Yes [2] (very low)
GLP-1 receptor activation (magnitude of response)	Yes (high)	-	-	No
Calcitonin release	Yes	Yes	No	No
↑ Calcitonin synthesis	Yes	Yes	-	-
C-cell hyperplasia	Yes	Yes	No	-
C-cell neoplasia	Yes	Yes	-	-

- indicates key events not evaluated in the stated species

[1]Published data indicate very low GLP-1 receptors in primary culture of human C-cells (69)

[2]Published data indicate 1/18 human thyroid samples have GLP-1 receptors in thyroid C-cells (62) and that 5/18 or 10/36 human MTCs have the GLP-1 receptor and the expression is confined to C-cells (63). No GLP-1 receptors were detected in primary culture of human C-cells (69)

[NDA206321 Rodent C-cell findings: Assessment of human relevance Oct 2013 P60]

The weight of evidence from mechanistic studies of liraglutide-induced thyroid C-cell tumors did not support the proposed mode-of-action in mice because:

1. Immunohistochemical localization and *in situ* hybridization studies of GLP-1Rs in thyroid did not adequately demonstrate the receptor protein or transcript were localized to calcitonin immunoreactive C-cells. A published study showed that thyroid from 60% of mice (3/5) were positive for GLP-1Rs detected by autoradiographic ligand binding, but GLP-1R binding activity wasn't localized to a specific cell-type.
2. Liraglutide caused focal C-cell hyperplasia, a preneoplastic lesion, without causing proliferation of normal C-cells (diffuse hyperplasia). These results indicate liraglutide transforms normal C-cells into preneoplastic C-cells in mice, a species lacking age-related increases in either plasma calcitonin or proliferative C-cell lesions.

The weight of evidence from mechanistic studies of liraglutide-induced thyroid C-cell tumors did not support the proposed mode of action in rats because:

1. Although published studies demonstrate GLP-1Rs in rat thyroid by autoradiographic tissue binding, GLP-1R agonist increased calcium-dependent calcitonin release from perfused rat thyroid cells, and inactivating the GLP-1R in mice reduces thyroid calcitonin transcript levels, the applicant's immunohistochemical and in situ hybridization studies did not conclusively demonstrate GLP-1Rs localized to C-cells.
2. Calcitonin was not a biomarker for liraglutide-induced thyroid tumors in rats, and there was no consistent, sustained effect of liraglutide on plasma calcitonin.
3. Liraglutide did not consistently increase thyroid calcitonin mRNA.
4. Liraglutide increased the incidence of age-dependent focal C-cell hyperplasia, but without accelerating its onset and without causing diffuse C-cell hyperplasia.

5. The incidence of liraglutide-induced thyroid C-cell tumors in rats increased with treatment duration, but required at least 7 months of drug exposure in both young and aged male rats. Therefore, liraglutide's tumorigenic effects were independent of the incidence of focal C-cell hyperplasia, which is higher in aged rats than in young rats.

Carcinogenicity studies in rats and mice, mechanistic studies of liraglutide-induced proliferative C-cell lesions, and clinical data were insufficient to conclude thyroid C-cell tumor findings in rodents were not relevant to human risk because:

1. Mechanistic studies did not adequately support the applicant's proposed novel mode of action for liraglutide-induced C-cell tumors in rats and mice.
2. After 26 to 28 weeks of treatment, liraglutide dose-dependently increased calcitonin in clinical study subjects, so if the proposed mode of action was correct, it may be operable in humans.

A large majority of members from the 2 April 2009 Endocrinologic and Metabolic Drug Advisory Committee convened to evaluate the efficacy and safety of liraglutide for the treatment of T2DM and CDER's Executive Carcinogenicity Committee (December 2008 meeting) both concluded there was insufficient evidence to dismiss human relevance of liraglutide-induced C-cell tumors in mice and rats. The approved label for Victoza includes a boxed warning that it is unknown if liraglutide causes thyroid C-cell tumors in humans and section "13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility" in the Victoza label states "*Human relevance of thyroid C-cell tumors in mice and rats is unknown and could not be determined by clinical studies or nonclinical studies [see Boxed Warning and Warnings and Precautions (5.1)].*"

Approval of Victoza NDA 22341 included 2 nonclinical post marketing requirements (PMRs) to further assess the risk of liraglutide-induced proliferative lesions of thyroid C-cells: PMR 1583-3, a 2-year study in mice to determine if 26 weeks of liraglutide treatment increases the lifetime risk of thyroid C-cell tumors, and PMR 1583-5, a 13-week mouse study to determine if liraglutide-induced focal C-cell hyperplasia depends on a thyroid glucagon-like peptide-1 receptor (GLP-1R) or activation of the rearranged-during-transfection (RET) proto-oncogene, a receptor tyrosine kinase constitutively activated by mutations in the RET gene in the majority of familial and sporadic MTCs in humans.

Victoza NDA 22341 PMR 1583-3 was fulfilled by study 210145, a 104-week study in CD-1 mice subcutaneously administered 0 (vehicle), 0.2, 1.0, or 3.0 mg/kg/day liraglutide once a day for 26 weeks followed by a 78 week recovery period. Liraglutide doses were selected based on results from the 104 week carcinogenicity study of 0, 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide in CD-1 mice (report 204229) that showed preneoplastic focal C-cell hyperplasia in thyroid occurred in males and females at $\geq$0.2 mg/kg/day, C-cell adenoma occurred in males and females at $\geq$ 1.0 mg/kg/day, and C-cell carcinoma occurred in females at 3.0 mg/kg/day. The 26-week duration of treatment was selected because that duration of treatment with up to 3.0 mg/kg/day liraglutide would be expected to induce preneoplastic C-cell focal hyperplasia, but not C-cell tumors in mice. The only distinction between C-cell focal hyperplasia and adenoma in rodents is the size of the lesion with foci of C-cells $\geq$5 average sized contiguous follicles considered adenomas. The table below summarizes the incidence of C-cell lesions occurring during the treatment period (study weeks 1 – 27) and during recovery (study weeks 28 – 106). By the end of the 26-week treatment period, liraglutide increased plasma calcitonin up to 14.1-fold in males at $\geq$0.2 mg/kg/day and up to 4.0-fold in females at $\geq$1.0 mg/kg/day and induced thyroid focal C-cell hyperplasia at $\geq$0.2 mg/kg/day in males (4.3% to 22.7% affected) and at 0.2 and 3.0 mg/kg/day in females (8.3% and 31.8%, respectively), but liraglutide did not induce C-cell tumors. By the end of the 78 week recovery period, plasma calcitonin remained elevated 1.4- to 1.8-fold in males previously treated with $\geq$0.2 mg/kg/day liraglutide, a low

incidence of thyroid focal C-cell hyperplasia exceeded the incidence in concurrent and historical control groups at 3.0 mg/kg/day liraglutide in males (3.8% affected at 3.0 mg/kg/day compared to 2.7% in concurrent controls and 0% in historical controls), and benign C-cell adenoma occurred in 1 female in the 3.0 mg/kg/day group (1.3% at 3 mg/kg/day liraglutide compared to 0% in concurrent and historical controls).

Incidence (% affected) of Thyroid C-cell Proliferative Lesions in Mice During the 26-week Treatment Period or 78-week Recovery Period in Study 210145

	Sex	Male				Female			
	Liraglutide Dose (mg/kg/day)	0	0.2	1	3	0	0.2	1	3
	Human Exposure Multiple[1]	-	1.8	10	43	-	1.8	10	43
Death / Termination Period	Thyroid C-cell Pathology								
Treatment (weeks 1 - 27)[2]	Focal Hyperplasia	0	4.3	8.3	22.7*	0	8.3	0	31.8*
Recovery (weeks 28 - 106)[3]	Focal Hyperplasia	2.7	0	1.3	3.8	0	0	0	0
	Adenoma	0	0	0	0	0	0	0	1.3

[1]Based on the $AUC_{0\text{-}24h}$ ratio using toxicokinetic data from the same doses used in the mouse carcinogenicity study and estimated steady state plasma liraglutide $AUC_{0\text{-}24h}$ 854 nM.hr in obese adult humans at the maximum recommended human dose of 3.0 mg/day liraglutide

[2]N = 22 - 25 mice/sex/dose examined

[3]N = 75 - 78 mice/sex/dose examined

*Statistically significantly different from control by pairwise comparison ($p < 0.5$)

Underlined value exceeds historical control group maximum for focal C-cell hyperplasia (0% M, 0.9% F) and C-cell adenomas (0% M, F)

Mice treated with 3.0 mg/kg/day liraglutide for 25% of their total life span (26 weeks) were at an increased risk of developing proliferative thyroid C-cell lesions (preneoplastic focal C-cell hyperplasia in males and benign C-cell adenoma in females) for up to 78 weeks after treatment was stopped. Due to the low incidence of proliferative C-cell lesions in thyroid in male and female high dose recovery group mice and in concurrent control group male mice, a clear relationship to liraglutide treatment was not established for proliferative C-cell lesions in high dose recovery groups. Whether or not transient exposure to liraglutide increases the lifetime risk of proliferative C-cell lesions in mice could not be adequately addressed by study 210145 because of the uncertainty that proliferative C-cell lesions in high dose recovery groups were related to liraglutide treatment. To date, results of this study have not been published.

Victoza NDA 22341 PMR 1583-5 was fulfilled by study 209306, a 13-week repeat dose study of 0 (vehicle), 0.03, 0.3, or 3 mg/kg/day liraglutide subcutaneously injected once a day in wild-type (WT) CD-1 mice and 0 or 3 mg/kg/day liraglutide subcutaneously injected once a day in GLP-1R-deficient (GLP1rKO) mice. Liraglutide doses in WT mice were selected based on previous studies showing proliferative thyroid C-cell changes in mice were not expected at 0.03 mg/kg/day liraglutide, C-cell hyperplasia was expected at 0.3 mg/kg/day, and C-cell hyperplasia was expected at 3 mg/kg/day with tumors developing during prolonged treatment (>26 weeks). To determine the requirement for functional GLP-1Rs to mediate liraglutide-induced proliferative C-cell lesions, GLP1rKO mice were treated with 3 mg/kg/day liraglutide, a tumorigenic dose. The 13-week treatment period was expected to be sufficient for inducing preneoplastic focal C-cell hyperplasia. Liraglutide-induced thyroid C-cell hyperplasia was GLP-1R-dependent in mice. Calcitonin immunohistochemical analysis of thyroid tissue sections to identify C-cells showed liraglutide increased the incidence of minimal to slight diffuse C-cell hyperplasia at $\geq$0.3

mg/kg/day in both male and female WT mice, but 3 mg/kg/day liraglutide did not cause C-cell hyperplasia in GLP-1rKO mice. At the end of treatment, plasma calcitonin was dose-dependently increased at ≥0.03 mg/kg/day liraglutide in WT mice (2.8- to 24.2-fold compared to controls in males and 2.5- to 12.1-fold in females), but liraglutide did not increase plasma calcitonin in GLP1rKO mice. The presence of functional GLP-1Rs in thyroid was confirmed in wild-type mice, but as expected, functional GLP-1Rs were absent from GLP1rKO mice. [^{125}I]GLP-1 bound to C-cells in thyroid tissue sections from female WT mice, but not from male or female GLP1rKO mice. Immunohistochemical analysis of thyroid tissue sections from WT mice in the control group without C-cell hyperplasia and 3 mg/kg/day liraglutide group with C-cell hyperplasia showed liraglutide induced phosphorylation of serine 235/236 in ribosomal protein S6 in normal and hyperplastic C-cells, but without inducing phosphorylation of tyrosine 1062 in RET or serine 217/221 in mitogen-activated protein kinase kinase 1 or 2 (MEK1/2). Liraglutide-induced thyroid C-cell hyperplasia and of ribosomal protein S6 activation were GLP-1R-dependent, but liraglutide did not activate RET or MEK1/2 in normal or hyperplastic C-cells. Results of this study were published (Madsen et al. Endocrinology. 2012 Mar;153(3):1538-47).

In Saxenda NDA 206321, the applicant submitted reports for 3 additional mechanistic toxicity studies: 2 studies in wild-type CD-1 mice and GLP-1rKO mice evaluating the effects of a single subcutaneous dose of exenatide (report 205207) or liraglutide (report 209188) on plasma calcitonin and an *in situ* ligand binding study evaluating GLP-1R localization in thyroid tissue sections from rats and humans (report CGo081003). Theses study reports were previously submitted in the 2009 annual report for IND 61040 investigating the use of liraglutide for the treatment of type 2 diabetes during the review of Victoza NDA 22341. In an autoradiographic ligand binding study evaluating GLP-1R binding activity in thyroid tissue sections from rats and humans, specific binding of the GLP-1R antagonist [^{125}I]exendin(9-39) occurred in thyroid tissue from rats (8/8), but not humans (0/13). Calcitonin immunohistochemical staining showed thyroid tissue samples from 3/13 humans were devoid of C-cells and C-cell density was lower in thyroid tissue sections from humans compared to rats. GLP-1R mediation of GLP-1R agonist-induced increased plasma calcitonin was evaluated using wild-type (WT) and GLP-1R knockout (GLP-1R KO) mice. Compared to WT mice, plasma calcitonin levels were higher and more variable in male GLP-1r KO mice, but not in females. In a single-dose study of subcutaneously injected 0, 1, or 5 mg/kg exenatide, exenatide increased plasma calcitonin levels in WT mice 6 hours after dosing, but the increase was not dose-related. Exenatide did not increase plasma calcitonin in GLP-1R KO mice. In a single-dose study of subcutaneously injected 0 or 1 mg/kg liraglutide, liraglutide increased plasma calcitonin 6 hours after dosing WT mice, but not GLP-1R KO mice. GLP-1R agonist-induced increased plasma calcitonin was GLP-1R-dependent in CD-1 mice.

To provide evidence that the key step of GLP-1R agonist-induced increased calcitonin secretion preceding liraglutide-induced thyroid C-cell proliferative lesions occurred in rats, the applicant provided report 409 SqA 2058 titled “Statistical analysis of the correlation between initial calcitonin change and focal C-cell hyperplasia and adenoma scores from studies NN204163, NN204310, and NN205119” evaluating the correlation between the change in plasma calcitonin levels during the first 28 days of treatment with terminal proliferative thyroid C-cell changes (focal C-cell hyperplasia or adenoma) in young (2 months old, treated for up to 69 weeks) or aged (8 months old treated for up to 43 weeks) male rats subcutaneously administered 0 (vehicle) or 0.75 mg/kg/day liraglutide. Statistical Review and Evaluation from Dr. Meiyu Shen in CDER’s Biometrics Division concluded the applicant’s analysis of the correlation between early calcitonin change during the first 28 days of treatment and terminal proliferative C-cell changes is neither useful nor appropriate for a nonclinical study. The absence of a persistent drug-related increase in plasma calcitonin secretion in liraglutide-treated rats does not support the applicant’s mode-of-action. A published study showed exenatide induced thyroid C-cell hypertrophy, increased C-cell density, and increased C-cell calcitonin content in Sprague Dawley rats subcutaneously injected with 0.01 mg/kg/day exenatide for 75

days (Bulchandani et al. Eur J Pharmacol. 2012 Sep 15;691(1-3):292-6), but circulating calcitonin levels were not assessed.

Since approval of Victoza NDA 22341for the treatment of T2DM in January 2010, 2 GLP-1R agonists were approved for marketing in the US (albiglutide (Tanzeum, BLA 125431), and exenatide long-acting release (LAR) (Bydureon, NDA 22-220), an extended release formulation of exenatide administered once a week) and lixisenatide was approved by the European Commission (Lyxumia). Exenatide LAR caused C-cell tumors in rats, but carcinogenicity was not assessed in mice. Lixisenatide caused C-cell tumors in mice and rats. Carcinogenicity of albiglutide was not assessed due to the development of drug-clearing anti-drug antibodies in rodents.

Several GLP-1R localization studies impacting assessment of human relevance of GLP-1R agonist-induced thyroid C-cell tumors were published, and most of these studies were funded, in whole or in part, by biopharmaceutical companies. GLP-1Rs were not detected in thyroid of monkeys or humans by immunohistochemical staining with an anti-GLP-1R monoclonal antibody or by in situ radioligand binding (Pyke et al, Endocrinology, April 2014, 155(4):1280–1290). *In situ* radioligand binding to thyroid tissue samples showed GLP-1R expression on normal C-cells in thyroid from mice, on normal, hyperplastic, and neoplastic C-cells in thyroid from rats, and in 27% (10/36) of medullary thyroid carcinoma tissue samples from humans, but not in non-neoplastic thyroid tissue from humans (0/6) (Waser et al, Neuroendocrinology 2011;94:291–301). In human thyroid tissue samples evaluated for GLP-1R immunoreactivity, 2/10 normal thyroid samples were GLP-1R positive (localized to C-cells), 3/3 multinodular goiter samples were GLP-1R positive (localized to C-cells), 11/12 medullary thyroid cancer samples were GLP-1R positive (localized to C-cells in 10/11 GLP-1R positive samples), 5/5 C-cell hyperplasia samples with RET germline mutations were GLP-1R positive (localized to C-cells), 4/4 reactive C-cell hyperplasia samples were GLP-1R positive (localized to C-cells), and 3/17 papillary thyroid cancer samples were GLP-1R positive (Gier et al, J Clin Endocrinol Metab. Jan 2012; 97(1): 121–131). In primary cultures of thyroid cells, the proportion of C-cells in primary rat thyroid cultures was ~0.4 (ranging from 0.1% to 1.0%) while the proportion of C-cells in human cultures was approximately 10-fold lower (~0.04%) and functional GLP-1Rs were Boess et al. J Mol Endocrinol. 2013 Apr 12;50(3):325-36).

The weight of evidence from GLP-1R localization studies is sufficient to conclude the receptor is expressed in thyroid C-cells in mice and rats and in some proliferative C-cell lesions in humans. The weight of evidence is sufficient to conclude GLP-1R agonist-induced proliferative changes in rodent thyroid C-cells are mediated by the GLP-1R. However, there is insufficient evidence to supports the applicant's proposed mode-of-action in mice and rats for the reasons previously stated, except GLP-1R expression occurs in C-cells from mice and rats and some proliferative C-cell lesions in humans. Although the weight of evidence indicates the density of C-cells in thyroid is lower in primates compared to rodents and normal primate C-cells do not express GLP-1Rs or only express low levels of GLP-1Rs, some human proliferative C-cell lesions express higher levels of GLP-1Rs. Furthermore, liraglutide was not a mitogen in rat C-cell lines CA77 and MTC 6-23 or in the human C-cell line TT (report 205295) suggesting other type of cells may be required for GLP-1R agonists-induced C-cell tumors.

Despite additional data, the proposed mode-of-action for liraglutide-induced thyroid C-cell tumors is not supported by current nonclinical data. There is insufficient evidence to conclude rodents are more sensitive than humans to GLP-1R agonist induced C-cell proliferative changes. Human relevance of liraglutide-induced thyroid C-cell tumors in mice and rats is unknown and has not been determined by nonclinical studies.

Pancreas Safety

Approval of Victoza NDA 22341 included Post Marketing Requirement (PMR) 1583-4 requiring the applicant to determine the effects of liraglutide on the exocrine pancreas in a rodent model of insulin-resistant type 2 diabetes mellitus. Study LoSi100801 titled "A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes" fulfilling PMR 1583-4 was reviewed under NDA 22341. Effects of liraglutide on the exocrine pancreas were characterized in a 3-month repeat subcutaneous dose toxicity study of 0 (vehicle), 0.4, or 1.0 mg/kg/day liraglutide administered once a day or 1.0 mg/kg/day administered twice a day (0.5 mg/kg/injection) to male and female diabetic ZDF *fa/fa* rats that included 0 (vehicle) and 1.0 mg/kg/day liraglutide (administered once a day) 4-week recovery groups, baseline diabetic ZDF *fa/fa* rats sacrificed prior to starting treatment, a 0 mg/kg/day group (liraglutide vehicle) of nondiabetic ZDF *+/?* rats (lean phenotype), and comparator groups treated by continuous subcutaneous infusion of 0 (vehicle) or 0.25 mg/kg/day exenatide. To minimize toxicity of high doses of liraglutide in diabetic rats, the dose of liraglutide was titrated over 7 to 16 days to the final dose and the dose titration phase was immediately followed by a 12-week treatment period. Treatment-related mortality occurred in 3/8 diabetic males in a control group found dead from an unknown cause within 4 days after they were mistakenly administered a single subcutaneous dose of 0.5 mg/kg liraglutide on day 17. Liraglutide (0.4 or 1 mg/kg/day) was pharmacologically active in diabetic ZDF *fa/fa* rats decreasing food and water consumption, decreasing body weight gain, lowering non-fasting plasma glucose, and lowering HbA1c at $\geq$ 0.4 mg/kg/day in males and females. Increased pancreas β cell mass in diabetic females at 0.4 or 1.0 mg/kg/day liraglutide, but not in diabetic males, was attributed to improved β cell survival and consistent with increased glucose lowering efficacy in females. At several time points during the 12-week treatment period, 0.4 or 1.0 mg/kg/day liraglutide increased group mean plasma p-amylase in male and female diabetic rats, but without increasing plasma lipase or plasma triglycerides and without evidence of treatment-related macroscopic or microscopic pathology findings in the exocrine pancreas at the end of the study. In male diabetic rats, liraglutide had no effect on pancreas weight. In diabetic females, relative pancreas weight was significantly decreased at $\geq$ 0.4 mg/kg/day liraglutide, but decreased pancreas weight lacked correlative quantitative or qualitative changes in the exocrine or endocrine pancreas. Liraglutide did not affect exocrine cell mass (acinar cells or ductal) or exocrine cell proliferation in male or female diabetic rats. Liraglutide had no adverse effects on the exocrine pancreas of diabetic ZDF *fa/fa* rats. Results of this study were published (Vrang et al. Am J Physiol Endocrinol Metab. 2012 Jul 15;303(2):E253-64).

In Saxenda NDA 206321, the applicant submitted study report 208304 evaluating microscopic pathology of pancreas from monkeys treated for 87 weeks with liraglutide and they submitted an evaluation of pancreas pathology findings from high dose group monkeys in repeat dose toxicity studies of liraglutide against diagnostic criteria for pancreatic intraepithelial neoplasia (PanIN). Report 208304 was previously submitted in a November 2008 annual report for IND 61040 investigating the use of liraglutide for the treatment of type 2 diabetes during the review of Victoza NDA 22341. In that study, microscopic examination of pancreas from monkeys treated for 87 weeks with 0 (vehicle), 0.25, or 5 mg/kg/day liraglutide for 87 weeks from study 203262 titled "Effects on calcium homeostasis related parameters after up to 87 weeks daily subcutaneous administration in male and female cynomolgus monkeys – combined evaluation of in life phase including thyroid histopathological evaluation" showed minimal focal inflammatory changes in the exocrine pancreas (characterized as perivascular inflammatory cell foci in 1 monkey, ductal inflammatory cell foci in a second monkey, and parenchymal inflammatory cell foci and focal lobular atrophy in a third) occurred in 3/5 males in the 5 mg/kg/day liraglutide group. In the absence of a consistent inflammatory effect in high dose group males or a similar effect in liraglutide-treated females, the relation to treatment was considered equivocal. Findings in pancreas of monkeys in 5 mg/kg/day liraglutide high dose

groups from 4 week (report 980184), 13 week (report 990191), and 52 week (report 200241) repeat dose toxicity studies and the 87 week mechanistic thyroid toxicity study (report 203262) were evaluated against diagnostic criteria for pancreatic intraepithelial neoplasia (PanIN) developed by the National Cancer Institute Pancreas Cancer Think Tank. There were no PanIN lesions present in monkeys treated with up to 5 mg/kg/day liraglutide for up to 87 weeks.

10.1 Local Toxicity

Study title: NNC 0090-0000-1170: Local toxicity 2 and 5 days after subcutaneous injections in the pig (report 208224)

Key study findings:

- Injection site histopathology findings of inflammation, hemorrhage, and fibroblast activation attributed to the vehicle persisted for at least 5 days after dosing.
- Study deficiencies:
  - The sex of pigs used in this study was not reported.

Summary and Conclusions

Local toxicity of the "to-be-marketed formulation" of liraglutide was evaluated in LYD pigs 2 and 5 days after subcutaneous injection of 0.2 mL 0 (vehicle), 0 (saline), or 6 mg/mL liraglutide (1.2 mg/injection liraglutide solution in vehicle, pH 8.15) in the dorsal surface (4 pigs total, sex not reported, 6 shaved marked injection sites behind the shoulder on each pig). The dose of liraglutide was 1.2 mg/injection. Injections on the left side were given on day 1 (5 days prior to termination) and on the right side on day 4 (2 days prior to termination). Table 1 shows the composition of saline, vehicle, and liraglutide solution for injection.

Table 1 Composition of NNC 0090-0000-1170, Vehicle, and Negative Control

Ingredients	NNC 0090-0000-1170	Vehicle	Saline (Fresenius Kabi AB)
Batch no.	TQ50297	TQ50655	06B27C25
Date of manufacture	05-Nov-2007	28-Oct-2007	-
Expiry date	-	-	Feb-2009
Liraglutide (NNC 0090-0000-1170)	6.0 mg/ml	-	-
Disodium phosphate, dihydrate	1.42 mg/ml	1.42 mg/ml	-
Phenol	5.5 mg/ml	5.5 mg/ml	-
Propylene glycol	14.0 mg/ml	14.0 mg/ml	-
(b) (4)			-
			-
NaCl			0.9 mg/ml
pH	8.15	8.15	-
Storage	+2°C to +8°C in the dark		Room temperature
(b) (4)			

[P10]

Pigs were anesthetized and injected using a NovoPen 3.0 device with a 28 gauge needle inserted 4 mm into the skin.

On study day 6, anesthetized pigs were sacrificed by exsanguination through the subclavian vein and/or artery and skin samples including subcutaneous tissue were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin for microscopic examination. Paraffin-embedded tissue sections, preferably containing the needle canal, were cut to a thickness of 5 μm, stained with hematoxylin and eosin, and examined microscopically. Histopathology findings were scored using a 5 point scale (minimal, slight,

moderate, marked, and pronounced). Two slides were prepared from each 2 mm tissue section, and there were 2 tissue sections per injection site. The reviewing pathologist examined at least 50% of the slides. Histopathology findings in the report are the result of consensus between the reviewing pathologist and the study pathologist. Study parameters were overall clinical signs, clinical signs at injection sites, body weight, and macroscopic and microscopic pathology at the injection site.

The following protocol deviations were noted, but none of the deviations affected interpretability of the study.

Table 2 Deviations from Study Plan

Activity	Day	Deviation
Injection of Torbugesic Vet.	2 and 4	Due to development of adverse clinical signs, pig no. 2, 3 and 4 received treatment (Torbugesic Vet.) to alleviate pain.
Body temperature measurement	1, 2 and 3	The body temperature was measured rectally. Pig no. 1-3 on day 1 and pig no. 4 on day 1, 2 and 3.
Blood glucose measurement	1 and 2	The blood glucose level was measured on blood obtained from the ear vein catheter. Pig no. 1 and 2 on day 1 and pig 4 on day 1 and 2.
Sedation	4	Prior to subcutaneous injection on Day 4, pigs were not anaesthetised using Rapinovet Vet., but adequately sedated and anaesthetised and pain relieved using a combination of Sedator and Torbugesic Vet.
Body weight	6	The body weight was also recorded on Day 6.
Exsanguination	6	Pentobarbital was not used prior to exsanguination. Pigs were killed by exsanguination after reaching adequate anaesthetic level.

[P10]

Clinical signs in all 4 pigs were subdued behavior or unwell, reduced appetite and erythema. Pigs 1 and 2 vomited on separate occasions, and pigs 2, 3, and 4 had signs of abdominal pain or discomfort and they were treated with an analgesic (Turbogesic vet) to alleviate their discomfort. Blood glucose and body temperature were unaffected by treatment.

Appendix C Individual Clinical Signs and Treatment

Observation	Animal Day of observation			
	1	2	3	4
Subdued, unwell	1, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4
Reduced appetite	2, 3, 4	2, 3, 4, 5, 6	2, 4, 5, 6	2, 3, 4, 5, 6
Vomited	1	4		
Erythema	1, 4	1, 2, 4, 5	1, 2, 4, 5	1, 2, 3, 4, 5
Signs of abdominal pain/discomfort		2, 4	2, 4	2
Treated with analgesic		2, 4	2, 4	2, 4*

* Injection given in error

[P32]

Decreased body weight between day -1 and day 6 correlated with reduced appetite. The contribution of anesthesia to effects on clinical signs and body weight are unknown and cannot be discerned using this study design because all pigs were treated with anesthetics and liraglutide.

Animal no.	Bodyweight kg		
	Day -14	Day -1	Day 6
1	43	53	-
2	45	54	53
3	44	56	53
4	44	57	55

- The animal was not weighed

[P31]

Clinical signs at the injection site were slight redness and swelling immediately after injection and swelling for up to 2 hours afterward with swelling persisting 2 hours after dosing related to injection with the vehicle, but not related to injection with liraglutide.

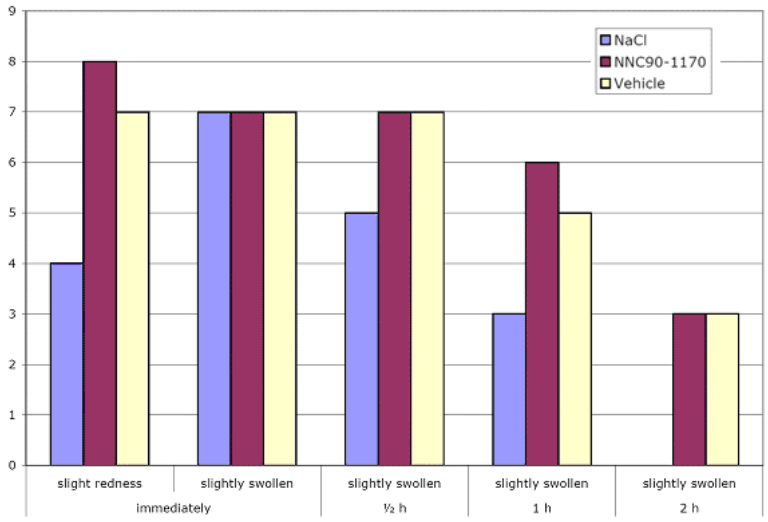


Figure 1 Local Clinical Reaction at Injection Sites

The total no. of injection sites per compound is 8

[P21]

There were no macroscopic injection site findings at necropsy 2 or 5 days after dosing. Microscopic injection site findings occurring 2 or 5 days after injection are summarized in Tables 3 and 4 below. Two days after dosing, the incidence or severity of minimal subcutaneous inflammatory cell infiltration, minimal to slight subcutaneous hemorrhage, minimal subcutaneous edema, congestion, and encrustation of the skin was increased in vehicle or liraglutide injection sites compared to saline injection sites. Five days after dosing, subcutaneous inflammatory cell infiltration and subcutaneous hemorrhage persisted and subcutaneous fibroblast activation emerged in vehicle or liraglutide injection sites, but not at saline injection sites.

Table 3 Histological Changes at Injection sites 2 Days after Administration

Diagnosis/Treatment	Saline	Vehicle	NNC 0090-0000-1170
No of injection sites examined	4	4	4
No abnormalities detected	2		
Inflammatory cell infiltration, subcutaneously			
Minimal	2	4	4
Haemorrhage, subcutaneously			
Minimal	2	1	3
Slight		2	1
Oedema, subcutaneously			
Minimal		3	2
Fibrin, subcutaneously			
Minimal	1	2	1
Congestion		1	1
Encrustation on the skin surface	1	2	3
Injection canal present	2	3	2

[P18]

Table 4 Histological Changes at Injection sites 5 Days after Administration

Diagnosis/Treatment	Saline	Vehicle	NNC 0090-0000-1170
No of injection sites examined	4	4	4
No abnormalities detected	4		
Inflammatory cell infiltration, subcutaneously			
Minimal			2
Slight		4	2
Haemorrhage, subcutaneously			
Minimal		4	2
Fibroblast activation, subcutaneously			
Minimal		3	2
Injection canal present		1	3

[P19]

Study title: Liraglutide: Local tolerance study in rabbits (report 208428)

Key study findings:

- Intramuscular injection of liraglutide or vehicle in rabbits did not cause clinical injection site reactions, but caused microscopic focal accumulation of mononuclear cells and skeletal muscle fiber degeneration / regeneration. The severity of muscle fiber degeneration / regeneration was greater at sites injected with liraglutide compared to vehicle.
- Intravenous injection of liraglutide or vehicle in rabbits caused a mild injection site reaction that resolved within 5 days after dosing. Injection site microscopic pathology findings 5 days after dosing were necrosis of the vessel wall, perivascular necrosis, hemorrhage, and accumulation of inflammatory cells in both vehicle and liraglutide injection sites.
- Intra-arterial injection of liraglutide or vehicle in rabbits caused clinical injection site reactions including swelling, erythema, and hemorrhage that were generally more severe in liraglutide-treated groups. Five days after dosing, macroscopic findings consistent with hemorrhage had correlative microscopic findings of perivascular hemorrhage, edema, and inflammation that were more pronounced at liraglutide-treated sites.

Summary and Conclusions

This study evaluated local toxicity of single doses of liraglutide injected in male albino New Zealand White (NZW) rabbits by routes not intended for clinical use: intramuscular (im), intravenous (iv), or intra-arterial (ia). Rabbits (4/group) were injected with 0.06 mL of 6 mg/mL liraglutide (clinical formulation, right side of body) or vehicle (left side of body). Rabbits received im injections in the thigh (group 1), iv injections in the ear (group 2), and ia injections in the ear (group 3). Macroscopic and microscopic pathology of injection sites was determined 5 days after administering injections.

Decreased body weight, food consumption, and water consumption were attributed to the pharmacological effects of liraglutide, and rabbits recovered from these effects by day 5. Group mean body weight ranged from 4.09 to 4.15 kg (Table 1). Based on an average body weight of 4.1 kg, the weight-based liraglutide dose was 0.09 mg/kg (0.36 mg/injection). Average body weight decreased 52 to 100 g from day 1 to day 5 with correlative decreased food consumption that recovered over the 5 days after dosing (Table 8).

Table 1 Body weight (g) - Group mean values

GROUP	DAY 1			DAY 3			DAY 5			BODY WT GAIN DAY 1 TO 5		
	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N
1	4089	323	4	3844	297	4	3989	271	4	-100	80	4
2	4124	256	4	3929	218	4	4071	180	4	-53	132	4
3	4153	207	4	3963	236	4	4102	233	4	-52	62	4

[P24]

Table 8 Food consumption – Individual values

GROUP	CAGE NO	DAY 2	DAY 3	DAY 4	DAY 5	TOTAL DAY 1 TO DAY 5
1	1	10	2	44	136	192
	2	136#	27	171	189	523#
	3	22	1	63	224	310
	4	1	12	124	161	298
2	5	15	19	170	224	428
	6	0	0	31	124	155
	7	3	45	156	197	401
	8	5	23	158	194	380
3	9	4	93	177	188	462
	10	16	13	79	166	274
	11	52	26	201	244	523
	12	2	11	85	162	260

Spillage of diet was registered on Day 2. It was evaluated that the animal had hardly eaten.

[P39]

The table below summarizes macroscopic and microscopic pathology findings at injection sites evaluated 5 days after dosing.

At intramuscular injection sites, there were no injection site reactions in vehicle or liraglutide treated groups. Five days after dosing, there were no macroscopic injection site findings at necropsy. Microscopic injection site findings were minimal focal accumulation of mononuclear cells (primarily macrophages) and/or regenerating / degenerating muscle fibers in

both vehicle and liraglutide-treated sites. The severity of degenerating / regenerating muscle fibers was slightly higher in at the liraglutide injection site (slight) compared to vehicle injection (minimal) in one rabbit.

At iv injection sites, clinical injection site reactions were slight to moderate hemorrhage with similar incidence and severity at vehicle and liraglutide-treated sites. Five days after dosing, there were no macroscopic pathology findings at necropsy. Microscopic minimal necrosis of the vessel wall, minimal perivascular necrosis, minimal hemorrhage, minimal accumulation of inflammatory cells, and minimal crust occurred in a few animals, but the incidence and severity were similar at vehicle and liraglutide-treated sites.

At intra-arterial injection sites, clinical injection site reactions were slight to marked hemorrhage that was more severe at liraglutide-treated sites, erythema (one vehicle-treated site and one liraglutide site), and swelling that was slightly more pronounced at liraglutide-treated sites. Five days after dosing, gross necropsy findings were black-brown/red discoloration of liraglutide-injection sites in 2 rabbits and reddened discoloration in both ears (vehicle and liraglutide-treated) from another rabbit. Microscopic perivascular hemorrhage, periarterial fibrosis and edema, and accumulation of inflammatory cells occurred in both vehicle and liraglutide-treated rabbits. Severity of perivascular hemorrhage and periarterial fibrosis and edema was higher in liraglutide-injection sites. Minimal focal necrosis of the arterial wall occurred in a liraglutide-treated ear.

				Thigh (im)		Ear (iv)		Ear (ia)	
			Injection Site (route)						
			Injected Article (Liraglutide (LGT) or Vehicle (V))						
Injection Site Finding		Relation of Finding to Injections Site	Severity (grade)	LGT	V	LGT	V	LGT	V
Macroscopic	red discoloration		present					1	1
	black-brown discoloration		present					2	
Microscopic	focal edema	introduction site	1					1	2
			3					1	
	perivascular hemorrhage	introduction site	1			2	2	0	0
			2					0	1
			3					1	
		tip	1			1			
	focal mononuclear cell infiltrate	introduction site	1	1	2				
		tip	1	0	1				
		proximal to tip	1	2					
	focal inflammatory cells	introduction site	1					2	1
			2					1	1
	perivascular inflammatory cells	introduction site	1			2	4		
	vessel wall necrosis	introduction site	1			1	2	0	
			3					1 (artery)	
	perivascular necrosis	introduction site	1			1	2		
	periarterial f brosis	introduction site	1					1	1
			3					1	
	crust	introduction site	1			2		1	0
			2						1
	skeletal muscle myofiber degeneration / regeneration	introduction site	1	1	1				
		tip	1	-	2				
		proximal to tip	1	0	1				
			2	1					

N = 4 rabbits/injection route, 1 liraglutide and 1 vehicle injection site/rabbit

10.2 Mechanistic Toxicity Studies, Thyroid Safety

Study title: Liraglutide: In-situ ligand binding on the GLP-1 receptor in rat and human thyroid (report CGo081003)

Key study findings:

- C-cell density was lower in tissue sections from human thyroid compared to rat thyroid. Thyroid samples from 3/13 humans were devoid of C-cells.
- Specific binding of [^{125}I]exendin(9-39) occurred in thyroid tissue from rats (8/8), but not humans (0/13).
- Cellular localization of GLP-1 receptors in rat thyroid was not adequately demonstrated by the data submitted.

Summary and Conclusions

This study evaluated functional GLP-1 receptor (GLP-1R) activity in thyroid tissue samples from rats and humans by autoradiographic ligand binding. Autoradiographic ligand binding to rat pancreas tissue sections, a positive control, showed islet-specific binding using [^{125}I]GLP-1(7-36)amide (0.3 nM), a radiolabeled GLP-1R agonist, or [^{125}I]exendin(9-39) (0.3 nM), a GLP-1R antagonist. [^{125}I]exendin(9-39) gave a higher signal-to-noise ratio and it was more chemically stable, so it was used in thyroid tissue autoradiographic ligand binding studies. Figure 1 (magnification unknown) shows an autoradiograph superimposed on H&E stained pancreas to demonstrate darker areas due to higher levels of radioligand were localized to islets. Pictures in the center show total binding and pictures on the right show nonspecific binding using negative images of autoradiograms.

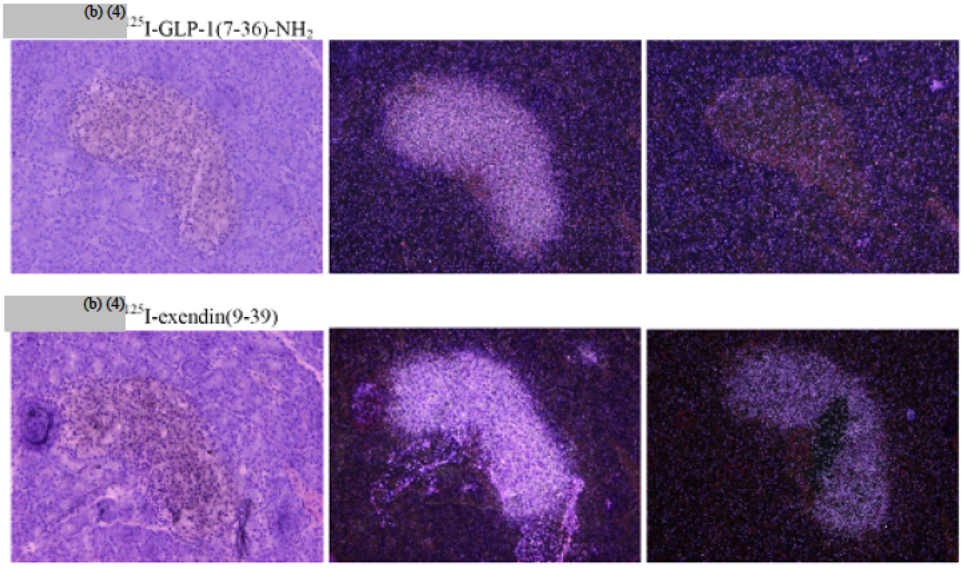


Figure 1 Rat pancreas ISLB with ^{125}I-GLP-1(7-36)-NH$_2$ or ^{125}I-exendin(9-39)

[P14]

Distribution of C-cells in rat and human thyroid was determined by anti-calcitonin staining using a polyclonal rabbit anti-human calcitonin antibody ((b) (4)). Regular sampling of rat thyroid tissue sections showed C-cells (stained brown) occurred in the central parafollicular area of the gland (Figure 2).

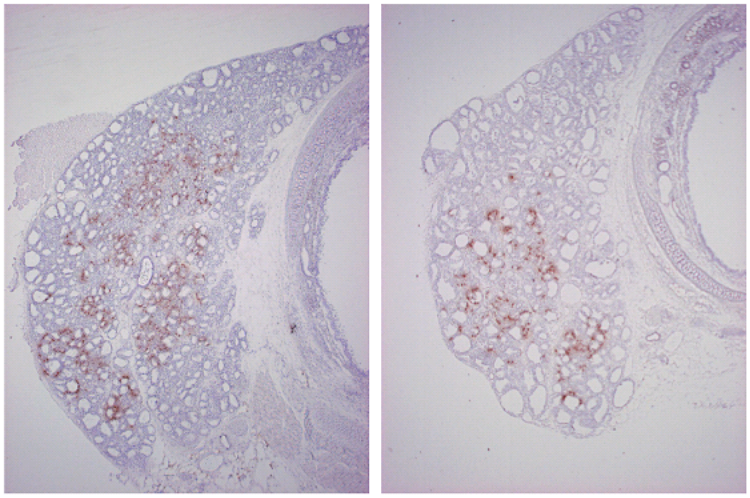


Figure 2 Calcitonin staining of C-cells distributed parafollicularly in the central area of the rat thyroid gland

[P15]

In thyroid tissue samples from 13 different humans (7 post-mortem, 6 surgical resections consisting of normal tissue adjacent to thyroid papillary carcinomas), C-cell density was generally much lower. Figure 3 shows calcitonin-immunoreactive cells (stained brown). Examining 6-10 thyroid tissue sections from each donor showed C-cell occurred in small groups (left, thyroid tissue from 2 humans) or as solitary cells (center, thyroid tissue from 8 humans), but thyroid tissues from 3/13 humans were devoid of C-cells (left).

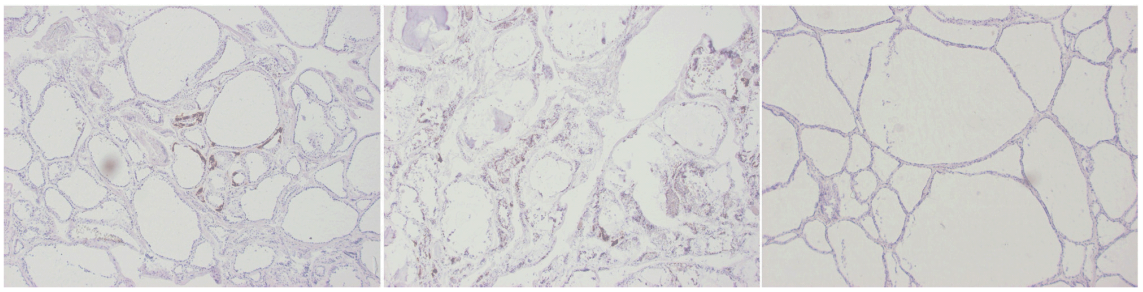
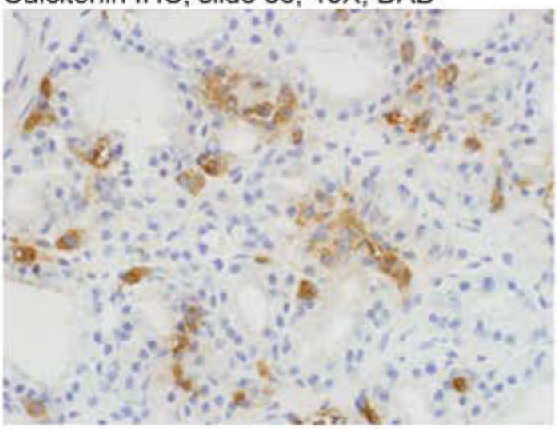


Figure 3 C-cell calcitonin staining in human thyroids

[P16]

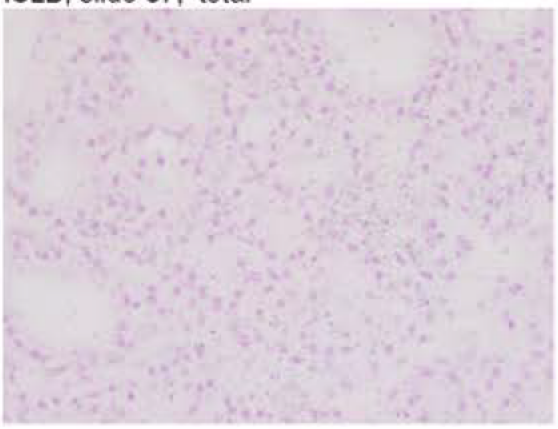
Autoradiographic ligand binding studies using rat and human thyroid tissue sections were performed with [^{125}I]exendin(9-39). No specific radioligand binding was detected in human thyroid. Figure 5 shows anti-calcitonin stained C-cells (brown) in rat thyroid tissue sections (image at top) and corresponding autoradiographs superimposed on images of the same H&E stained tissue sections with radioactivity quantified by counting the number of “silver grains”. By including a radioactivity standard on each slide, the number of silver grains / mm^2 could be converted to dpm / mg tissue, then fmol [^{125}I]exendin(9-39) / mg tissue. The density of exposed silver grains was only determined in rat thyroid tissue sections with higher densities of C-cells. [^{125}I]exendin(9-39) specifically bound to thyroid tissue samples from 8/8 rats. Mean silver grain density was 19,174 / mm^2 thyroid tissue (range 2,714 – 41,509) yielding estimated radioactivity concentrations of 1699 dpm/mg tissue (range 240 – 3,677) or 0.29 fmols specifically bound [^{125}I]exendin(9-39) / mg tissue.

Calcitonin IHC, slide 36, 40X, DAB

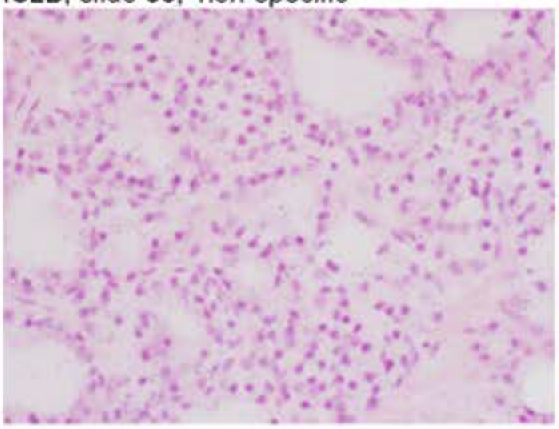


C-cells ~10% of cell area, 9% of total area

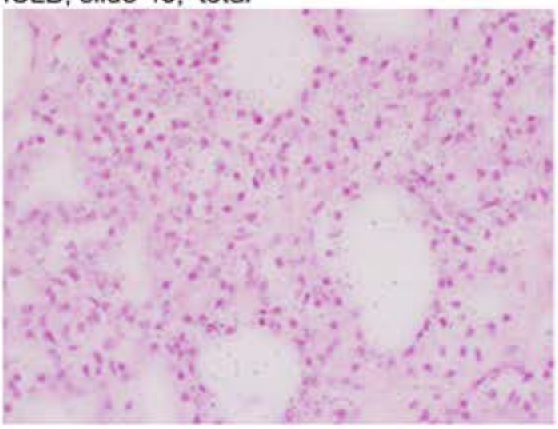
ISLB, slide 37, 'total'



ISLB, slide 38, 'non-specific'



ISLB, slide 40, 'total'



ISLB, slide 39, 'non-specific'

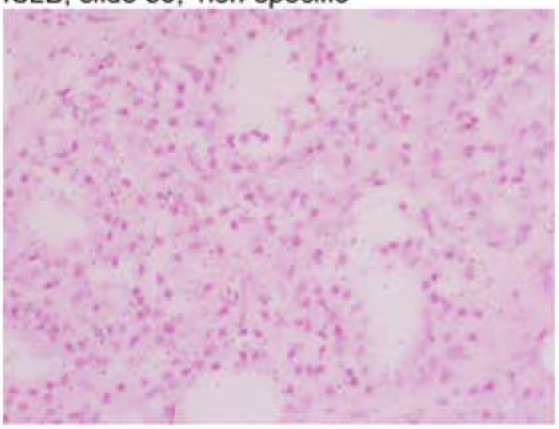


Figure 5 Identification of C-cell rich areas for ISLB quantitation in rat thyroid

The top picture shows the calcitonin staining C-cells, the lower pictures show corresponding tissue areas of lightly H-E stained ISLB sections on adjacent slides in the order of 'total', 'non-specific', non-specific', and 'total'. Silver grains in the sections are barely noticeable at this magnification. The fraction occupied by C-cells was determined by image analysis as brown area/total area.

[P22]

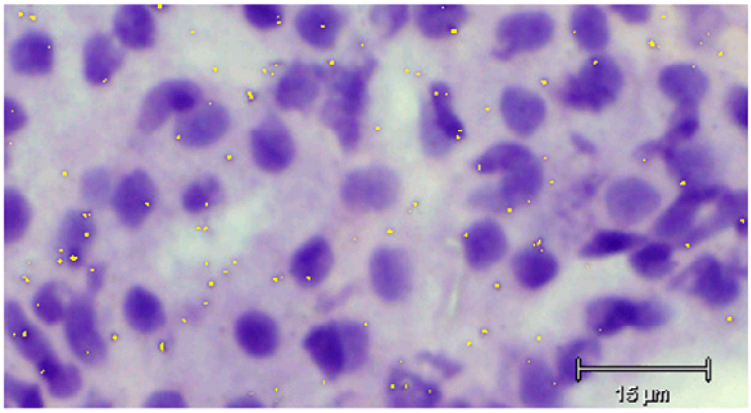


Figure 6 Identification of silver grains in bright field picture from ISLB
The picture shows the number of silver grains identified in the H-E picture by the technique of thresholding the result of identifying the maximum areas of regional contrast in the green channel of the RGB picture

[P23]

Study title: NNC 0113-0000-0000: Study of the acute effects on plasma calcitonin after a single subcutaneous administration in fasted GLP-1-receptor knockout mice and CD-1 mice (report 205207)

Key study findings:

- Exenatide-induced elevated plasma calcitonin was GLP-1 receptor-mediated in mice.
- GLP-1 receptor inactivation elevated basal plasma calcitonin in male mice.
- Study deficiencies were:
  - Altered genotype and the absence of functional GLP-1 receptors in GLP-1 receptor knockout mice was not demonstrated.
  - The source of GLP-1R knockout mice (GLP-1R KO) was (b) (4) but a GLP-1R KO genotype was not listed in the (b) (4) knockout mouse database (only GLP-2 receptor knockout mice were listed).
  - Cross reactivity of Immutopics rat calcitonin immunoradiometric assay with mouse calcitonin was not demonstrated.

Summary and Conclusions

Fasted wild type (WT) CD-1 mice (groups 2, 4, 6) or GLP-1 receptor (GLP-1R) knockout (GLP-1R KO) mice (groups 1, 3, 5, from (b) (4) were administered a single subcutaneous injection (5 mL/kg) of vehicle (groups 1 and 2) or exenatide at 1 mg/kg (groups 3 and 4) or 5 mg/kg (groups 5 and 6). Blood samples (retro-orbital) for measuring plasma calcitonin were collected 6 hours after dosing. Mice were 8 – 9 weeks old at the start of dosing. The design of the study is summarized in the table below.

Group	Animal strain	Dose level (mg/ml)	Number of animals	Animal numbers	
				Males	**Females**
1	GLP-1r KO	0	16	1-5	101-111
2	CD-1		16	6-10	112-122
3	GLP-1r KO	1	16	11-15	123-133
4	CD-1		16	16-20	134-144
5	GLP-1r KO	5	16	21-25	145-155
6	CD-1		16	26-30	156-166

[P15]

Plasma calcitonin in mice was measured using a commercial two-site immunoradiometric assay (IRMA from Immutopics Inc.) for rat calcitonin. The rat calcitonin IRMA used 2 different anti-calcitonin antibodies; a bead-immobilized monoclonal antibody to capture calcitonin and a purified ^{125}I-labeled polyclonal antibody to label it. Cross-reactivity of the assay with authentic mouse calcitonin was not reported, but the applicant states the use of this assay to quantify mouse calcitonin was validated. Assay calibration curves were constructed using rat calcitonin. The lower limit of quantification (LLQ) was 2 pg/mL rat calcitonin equivalents.

Initial body weight of GLP-1R KO mice (average body weight 28.6 g males and 23.6 g females) was lower compared to WT mice (average body weight 32.3 g males, 26.9 g females). There were no treatment-related clinical signs or mortalities.

The summary table (below) shows group mean plasma calcitonin (rat calcitonin equivalents, pg/mL) in wild type (WT) or GLP-1R knockout (GLP-1R KO) mice injected with a single subcutaneous dose of 0 (vehicle), 1, or 5 mg/kg exenatide. The 2 graphs below show all individual calcitonin measurements (individual symbols) and group mean (line) in wild type (WT) mice and GLP-1R KO (KO) mice treated with vehicle (0), 1 mg/kg exenatide (1), or 5 mg/kg exenatide. Plasma calcitonin was above the LLQ (2 pg/mL) in all plasma samples. GLP-1R inactivation increased basal levels of calcitonin in male mice. Compared to vehicle-treated WT mice, plasma calcitonin was higher in GLP-1R KO male and female mice, but the levels were only statistically significantly higher in males ($p < 0.05$ compared to vehicle-treated WT males).

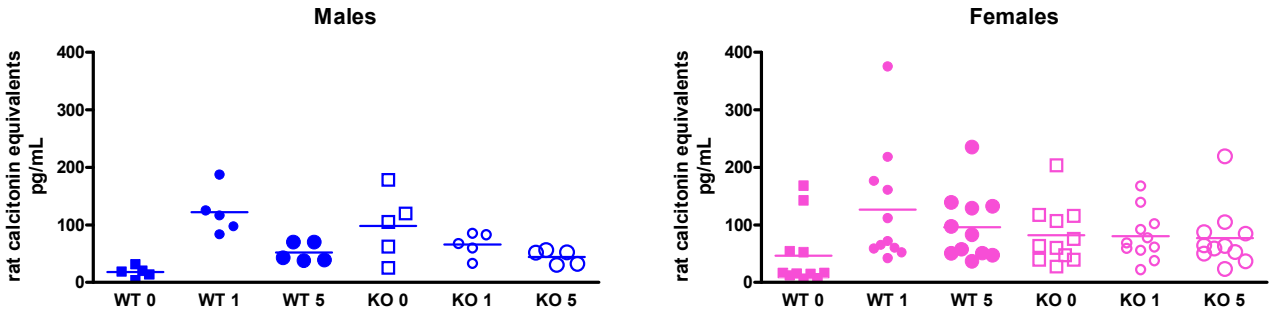
Plasma calcitonin (rat calcitonin equivalents, pg/mL) 6 hours after dosing in wild-type (WT) and GLP-1 receptor knock out (GLP-1R KO) mice

Treatment		WT		GLP-1 KO	
		M	F	M	F
Vehicle	Mean	18.1	46.4	98.4	81.8
	SD	10.0	56.8	58.1	51.4
	N	5	11	5	11
1 mg/kg exenatide	Mean	122.1*	126.7*	65.8	80.3
	SD	40.0	101.3	21.0	43.0
	N	5	11	5	11
	Fold Change from Vehicle	6.7	2.7	0.7	1.0
5 mg/kg exenatide	Mean	52.0*	96.3	44.6	76.8
	SD	16.8	59.5	12.3	52.6
	N	5	11	5	11
	Fold Change from Vehicle	2.9	2.1	0.5	0.9

*Statistically significantly different from vehicle control ($p < 0.05$, unpaired t-test)

Six hours after dosing, 1 mg/kg exenatide significantly increased plasma calcitonin 6.7-fold and 2.7-fold compared to vehicle-treated controls in wild-type male and female mice, respectively. Exenatide's effect on plasma calcitonin was not dose-related and the increase at 5 mg/kg exenatide was only statistically significant in males. Because plasma calcitonin levels were

higher and more variable in male GLP-1R KO mice, calcitonin in male GLP-1R KO mice was not significantly different from WT males treated with 1 mg/kg exenatide. There was a dose-related trend for exenatide to decrease plasma calcitonin in male GLP-1R KO mice, but differences between exenatide and vehicle control groups never reached statistical significance.



These results indicate GLP-1Rs mediate exenatide-induced increased plasma calcitonin because exenatide elevated plasma calcitonin in WT mice, but not in GLP-1R KO mice. In WT female mice, plasma calcitonin levels in some mice treated with 1 mg/kg exenatide were similar to vehicle treated mice suggesting they did not respond to exenatide. A published autoradiographic ligand binding study of GLP-1Rs in mice showed thyroids from 40% of CD-1 mice were GLP-1R negative (Korner et al, 2007 (PMID: 17475961)).

Study title: NNC 0090-0000-1170: Study of the acute effects on plasma calcitonin after a single subcutaneous administration in fasted GLP-1-receptor knockout mice and CD-1 mice (report 209188)

Key study findings:

- Liraglutide-induced increased plasma calcitonin was GLP-1 receptor-dependent in mice.
- GLP-1 receptor inactivation elevated basal plasma calcitonin in male mice, but not in females.
- Study deficiencies were:
  - Altered genotype and the absence of functional GLP-1 receptors in GLP-1 receptor knockout mice was not demonstrated.
  - The source of GLP-1R knockout mice was [(b) (4)], but a GLP-1R KO genotype was not listed in the [(b) (4)] knockout mice database (only GLP-2 receptor knockout mice were listed).
  - Cross reactivity of Immutopics rat calcitonin immunoradiometric assay with mouse calcitonin was not demonstrated.

Summary and Conclusions

Fasted wild type (WT) CD-1 mice (8/sex/group in groups 2 and 4 or 4/sex/group 6) or GLP-1R knockout (GLP-1R KO, CD-1 background) mice (8/sex/group, groups 1 and 3 or 4/sex/group 5) from [(b) (4)]) 6 – 7 weeks old at start of dosing were administered a single subcutaneous injection of vehicle (groups 1 and 2, 3.33 mL/kg), 1 mg/kg liraglutide (groups 3 and 4, 3.33 mL/kg), or a single ip injection of 41 mg/kg calcium gluconate (groups 5 and 6, 4.54 mL/kg). Blood samples (retro-orbital) for measuring plasma calcitonin were collected 6 hours after dosing with vehicle or liraglutide or 15 minutes after dosing with calcium. The study design is summarized in Table 2, below.

Table 2 Study Design

Group	Mouse Strain	NNC 0090 -0000-1170 (mg/kg)	Calcium (mg/kg)	Dose Volume (ml/kg)	Fasting (Hours Prior to Dosing)	Animal Numbers		Colour Code
						Males	Females	
1	GLP-1r KO	0 (vehicle)	None	3.33	1-1.33 h	1-8	101-108	White
2	CD-1					21-28	121-128	
3	GLP-1r KO	1	None	3.33	1-1.33 h	9-16	109-116	Blue
4	CD-1					29-36	129-136	
5	GLP-1r KO	None	40.86	4.54	6.75-7 h	17-20	117-120	Red
6	CD-1					37-40	137-140	

[P13]

As in the previous study, plasma calcitonin was measured using a commercial two-site immunoradiometric assay (IRMA from Immutopics Inc.) for rat calcitonin, but the lower limit of quantification (LLQ) in this study was 15 pg/mL rat calcitonin equivalents.

Initial body weight of GLP-1R KO mice (14.6 – 20.1 g males, 13.1 – 18.5 g females) was lower compared to WT mice (25.1 – 29.0 g males, 21.1 – 26.2 g females). There were no liraglutide-related clinical signs or mortalities. The table below shows group mean plasma calcitonin, excluding samples below the LLQ (group 2 males and group 3 females) or samples that did not contain enough plasma (group 5).

The summary table (below) shows group mean plasma calcitonin (rat calcitonin equivalents, pg/mL) in wild type (WT) or GLP-1R knockout (GLP-1R KO) mice injected with a single dose of vehicle (subcutaneous), 1 mg/kg liraglutide (LGT, subcutaneous), or 41 mg/kg calcium (Ca, ip). A value of 15 pg/mL was imputed for calcitonin assay measurements below the LLQ.

Plasma calcitonin (rat calcitonin equivalents, pg/mL) 6 hours after dosing (vehicle or liraglutide) or 0.25 hours after dosing (calcium) in wild-type (WT) and GLP-1 receptor knock out (GLP-1R KO) mice

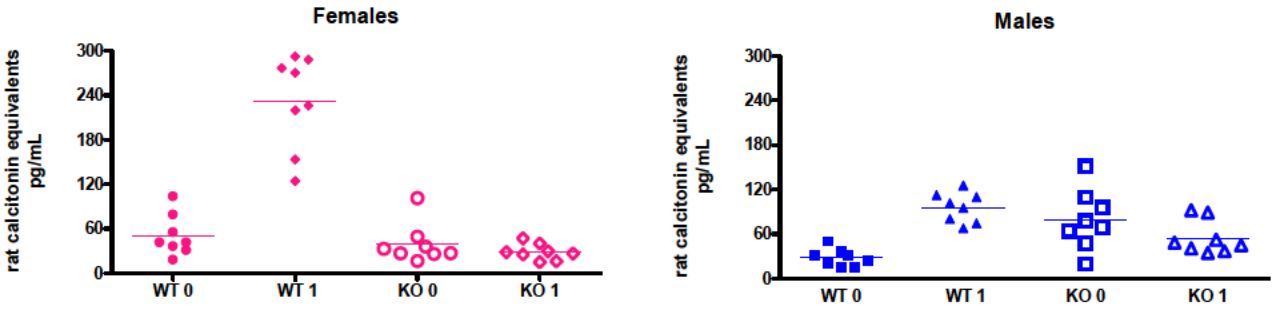
Treatment		WT		GLP-1 KO	
		M	F	M	F
Vehicle	Mean	28.3	51.1	79.8	39.5
	SD	12.1	27.9	40.1	26.7
	N	8	8	8	8
1 mg/kg LGT	Mean	96.2*	231.6*	55.2	28.7
	SD	20.1	63.5	22.7	10.9
	N	8	8	8	8
	Fold Change from Vehicle	3.4	4.5	0.7	0.7
41 mg/kg Ca	Mean	713.2*	1029.5*	1899.2*	1561.5*
	SD	468.1	283.1	864.6	793.3
	N	4	4	4	3
	Fold Change from Vehicle	25.2	20.1	23.8	39.5

*Statistically significantly different from vehicle control ($p < 0.05$, unpaired t-test)

GLP-1R inactivation increased basal plasma calcitonin in male mice, but not in females. Group mean plasma calcitonin was significantly higher in vehicle-treated GLP-1R KO males compared

to vehicle-treated WT males ($p < 0.005$). Plasma calcitonin in vehicle-treated GLP-1R KO mice and liraglutide-treated WT mice were not significantly different. To show both WT and GLP-1R KO mice were responsive to stimuli that normally increase plasma calcitonin, mice were intraperitoneally injected with calcium gluconate. Compared to vehicle-treated mice, calcium gluconate significantly increased plasma calcitonin 15 minutes after dosing in both WT mice (20-fold in females, 25-fold in males) and GLP-1R KO mice (40-fold in females, 24-fold in males).

Six hours after dosing, liraglutide significantly increased plasma calcitonin in wild-type male and female mice 3.4-fold and 4.5-fold, respectively, compared to vehicle-treated controls. Liraglutide did not increase plasma calcitonin in GLP-1R KO mice. In WT mice, plasma calcitonin in vehicle-treated males and females was not significantly different, but liraglutide elicited significantly higher calcitonin levels in females compared to males ($p < 0.0001$). However, calcitonin levels in all liraglutide treated males (range 68 – 126 pg/mL calcitonin) and females (range 125 – 293 pg/mL calcitonin) were above the range in vehicle-treated males (range 15 – 51 pg/mL calcitonin) and females (range 18 – 104 pg/mL calcitonin) suggesting they all responded to liraglutide. The 2 graphs below show all individual calcitonin measurements (individual symbols) and group mean (line at average value) in wild type (WT) mice and GLP-1R KO (KO) mice treated with vehicle (0) or 1 mg/kg liraglutide (1). In a published autoradiographic ligand binding study of GLP-1Rs in mouse thyroid, thyroids from 40% of CD-1 mice were GLP-1R negative (Korner et al, 2007 (PMID: 17475961)).



These results indicate GLP-1Rs mediate liraglutide-induced increased plasma calcitonin because liraglutide elevated plasma calcitonin in WT mice, but not GLP-1R KO mice. Inactivating the GLP-1R increases plasma calcitonin levels in male mice, but not in females.

10.3 Pancreas Safety

Study title: Histopathology extension to (b) (4) study no. 506326 (NNC 90-1170 (liraglutide) investigative subcutaneous toxicity study in cynomolgus monkeys (report 208304, GLP compliance claimed)

and

Liraglutide: Evaluation of pancreas from high dose animals from toxicity studies in cynomolgus macaques against diagnostic criteria for PanIN

Key study findings:

- Minimal focal inflammation in the exocrine pancreas occurred in 3/5 male cynomolgus monkeys treated with 5 mg/kg/day liraglutide for 87 weeks.

  - Report 208304 was originally submitted to IND 61040 investigating the use of liraglutide for the treatment of type 2 diabetes as part of an annual report in November 2008, while Victoza NDA 22341 was under review.
- There were no PanIN lesions present in monkeys treated with up to 5 mg/kg/day liraglutide for up to 87 weeks.

Summary and Conclusions

Pancreas from cynomolgus monkeys treated with 0 (vehicle), 0.25, or 5 mg/kg/day liraglutide (5/sex/dose) for 87 weeks in study 203262 titled "Effects on calcium homeostasis related parameters after up to 87 weeks daily subcutaneous administration in male and female cynomolgus monkeys – combined evaluation of in life phase including thyroid histopathological evaluation" were microscopically examined. At the end of the 87-week treatment period, pancreas was collected, fixed in 10% neutral buffered formalin, embedded in paraffin wax, and stored for possible future examination. For microscopic examination, pancreas tissue sections (~5 μm thick) were stained with hematoxylin and eosin and evaluated by the study pathologist by light microscopy. Findings were peer reviewed (by (b) (4) pathologists) and the results presented are the consensus of the study and reviewing pathologists (Table 1, below). Minimal focal inflammatory changes in the exocrine pancreas occurred in 3/5 males in the 5 mg/kg/day liraglutide group. In the exocrine pancreas of males in the 5 mg/kg/day liraglutide group, minimal perivascular inflammatory cell foci occurred in monkey 15M, minimal ductal inflammatory cell foci occurred in monkey 11M, and minimal parenchymal inflammatory cell foci and minimal focal lobular atrophy occurred in monkey 14M. Female 27F in the 5 mg/kg/day liraglutide group had minimal focal lobular atrophy in the exocrine pancreas, but it occurred in the absence of inflammation.

Table 1 Summary of Histology Findings

HISTOLOGICAL FINDINGS	GROUP DOSE	GROUP TOTALS Males Grp 1	Males Grp 2	Males Grp 3	Females Grp 1	Females Grp 2	Females Grp 3
ENDOCRINE SYSTEM							
PANCREAS (ENDOCRINE)		(5)	(5)	(5)	(5)	(4)	(5)
No abnormality detected		4	5	5	5	4	5
Haemangiectasis, islets of Langerhans, focal							
mild		1	0	0	0	0	0
Total Incidence		1	0	0	0	0	0
ALIMENTARY SYSTEM							
PANCREAS (EXOCRINE)		(5)	(5)	(5)	(5)	(4)	(5)
No abnormality detected		5	5	2	5	4	4
Lobular atrophy, focal							
minimal		0	0	1	0	0	1
Total Incidence		0	0	1	0	0	1
Inflammatory cell foci, perivascular							
minimal		0	0	1	0	0	0
Total Incidence		0	0	1	0	0	0
Inflammatory cell foci, ductal							
minimal		0	0	1	0	0	0
Total Incidence		0	0	1	0	0	0
Inflammatory cell foci, parenchymal							
minimal		0	0	1	0	0	0
Total Incidence		0	0	1	0	0	0

Figures in brackets represent the number of animals from which this tissue was examined microscopically

[P12-13]

The applicant contends due to the minimal severity, focal characteristic, and background incidence ranging from 0 – 30%, focal inflammation in pancreas of male monkeys in the 5 mg/kg/day liraglutide group were considered incidental.

Findings in pancreas of monkeys in high dose groups from 4 week (report 980184), 13 week (report 990191), and 52 week (report 200241) repeat dose toxicity studies and an 87 week thyroid safety mechanistic toxicity study (report 208304) were evaluated against the classification of duct lesions developed by the National Cancer Institute Pancreas Cancer Think Tank (see below).

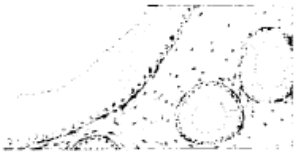
NCI Pancreas Think Tank Definitions

Normal ductal/ductular epithelium: cuboidal to low-columnar epithelium with amphophilic cytoplasm.

Squamous (transitional) Metaplasia: A process in which the normal cuboidal ductal epithelium is replaced by mature squamous or transitional epithelium without atypia.

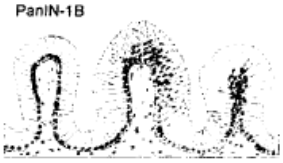
PanIN-1A: (Pancreatic Intraepithelial Neoplasia 1-A): flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. Nuclei are small and round to oval in shape. When oval the nuclei are oriented perpendicular to the basement membrane.

PanIN-1A



PanIN-1B: (Pancreatic Intraepithelial Neoplasia 1-B): papillary, micropapillary or basally pseudostratified architecture, but otherwise identical to PanIN-1A.

PanIN-1B



PanIN-2: (Pancreatic Intraepithelial Neoplasia 2): mucinous epithelial lesions may be flat or papillary with some nuclear abnormalities, such as loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. Mitoses are rare, but when present are non-luminal (not apical) and not atypical. True cribriforming luminal necrosis and marked cytologic abnormalities are not seen.

PanIN-2

PanIN-3: (Pancreatic Intraepithelial Neoplasia 3): papillary or micropapillary, may rarely be flat. True cribriforming, budding off of small clusters of epithelial cells into the lumen and luminal necroses. Loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented towards the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses which may occasionally be abnormal, nuclear irregularities and prominent (macro) nucleoli.

PanIN-3

[P2 -3]

There were no PanIN lesions present in monkeys treated with up to 5 mg/kg/day liraglutide for up to 87 weeks.

Histology Findings

Study Details	Animal Number	Diagnosis
28 Day tox Study (b) (4) Reference 571290 Novo Nordisk Reference 980184	10, 11, 12, 22, 23, 24	No PanIN lesions present
13 week tox study (b) (4) Reference 573036 Novo Nordisk reference 990191	13, 14, 15, 16, 19, 20, 33, 34, 35, 36, 39, 40	No PanIN lesions present
52 week tox Study (b) (4) Reference 577863 Novo Nordisk Reference 200241	13, 14, 15, 16, 19, 20, 33, 34, 35, 36, 39, 40	No PanIN lesions present
87 week mechanistic study (b) (4) Reference 514997 Novo Nordisk Reference 208304	11, 12, 13, 14, 15, 26, 27, 28, 29, 30	No PanIN lesions present

[P3]

11 Integrated Summary and Safety Evaluation

Liraglutide is a lipidated glucagon-like peptide 1 (GLP-1) analog with a prolonged duration of action compared to GLP-1 due to self-association at subcutaneous injection sites that reduces the rate of absorption, resistance to GLP-1 inactivating peptidases (DPP-4 and NEP), and increased binding to plasma proteins that increases protease resistance and reduces renal excretion. In January 2010, liraglutide was approved as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus at doses up to 1.8 mg/day administered as a single subcutaneous injection once a day under the brand name Victoza. Novo Nordisk is developing 3.0 mg/day liraglutide subcutaneously injected once a day as an adjunct to a reduced calorie diet and increased physical activity for chronic weight management in overweight adults with 1 weight-related comorbidity or obese adults under the brand name Saxenda. Both Saxenda and Victoza drug products consist of a rubber stoppered glass vial cartridge containing 3 mL of 6 mg/mL liraglutide solution provided in a pre-filled pen injector, The Saxenda PDS290 pen injector is capable of delivering a dose volume up to 0.5 mL in a single injection (3.0 mg liraglutide, the only proposed daily maintenance dose for weight management) while the Victoza pen injector can deliver a maximum dose volume of 0.3 mL (1.8 mg liraglutide the daily maximum recommended human dose (MRHD) for treatingT2DM).

Because clearance of liraglutide increases with body weight in humans, steady state systemic exposure in obese adults administered 3.0 mg/day liraglutide was similar to steady state systemic exposure in healthy adults administered 1.8 mg/day liraglutide, based on plasma liraglutide AUC comparison. Therefore, human safety margins and human exposure multiples for findings in nonclinical safety studies of liraglutide at the MRHDs of 1.8 mg/day liraglutide for the treatment of T2DM and 3.0 mg/day liraglutide for weight management are similar (based on AUC comparison), despite a 1.7-fold higher dose for weight management (see tabulated summary of safety margins in pivotal toxicity studies, below).

Species	Study Duration / Doses (mg/kg/day)	NOAEL (mg/kg)	Toxicity Defining the NOAEL	Animal Exposure @ NOAEL		Human Safety Margin (Based on AUC Comparison)[A]	
				Cmax (nM)	AUC (nM.hr)	1.8 mg/day liraglutide	3.0 mg/day liraglutide
CD-1 mice	13 weeks / 0.2, 1, 5	< 0.2	thyroid C-cell focal hyperplasia and ultimobranchial cysts	< 196	< 1959	< 2.4	< 2 3
	104 week carcinogenicity / 0 03, 0 2, 1, 3	Non-neoplastic: < 0.03	thyroid inflammatory cell infiltrate, femorotibial joint degradation, seminal vesicle lymphocyte infiltration, thymus tubular cystic hyperplasia, pigment accumulation from mild hemolysis	< 20	< 185	< 0.2	< 0 2
		Neoplastic: 0.2	C-cell tumors @ 1 mg/kg (preneoplastic focal hyperplasia @ ≥ 0 2 mg/kg)	141	1501	1 9	1 8
		Neoplastic, local: 1	dorsal skin and subcutis (surface for injections) fibrosarcomas @ 3 mg/kg	NA[B]	NA[B]	0 03[C]	0.03[C]
SD rat	26 weeks / 0.1, 0.25, 1	0.25	clinical signs, exocrine pancreas acinar cell hypertrophy with focal inflammation in females @ 1 mg/kg	107	1585	2 0	1 9
	104 week carcinogenicity / 0.075, 0.25, 0.75	Non-neoplastic: < 0.075	clinical signs (F) @ ≥ 0.075, thyroid C-cell focal hyperplasia @ ≥ 0.075 M, ≥ 0.25 F	< 31	< 423	< 0.5	< 0 5
		Neoplastic: < 0.075	thyroid C-cell tumors	< 31	< 423	< 0.5	< 0 5
	fertility / 0.1, 0.25, 1	Males : 1	No effect on reproductive parameters or abnormalities in sperm, but rel wt of some reproductive organs altered by treatment	577[D]	9074[D]	11	11
		Females: 0.25	early embryonic deaths @ 1 mg/kg	214	2693	3 3	3 2
	embryofetal development / 0.1, 0.25, 1	Maternal: 0.25	clinical signs @ 1 mg/kg	214	2693	3 3	3 2
		Fetal: < 0.1	fetal abnormalities	< 75[E]	< 691[E]	< 0.9	< 0 8
	pre- & post-natal / 0.1, 0 25, 1	F_0 reproductive: < 0.1	gestation delayed to day 22	< 75	< 691	< 0.9	< 0 8
		F_1 development: < 0.1	lower body weight compared to controls starting lactation day 7, agitated behavior in males descended from 1 mg/kg treated F_0 females.	< 75[F]	< 691[F]	< 0.9[F]	< 0 8
		F_1 reproductive: 1	none	1241[F]	9211[F]	11	11
		F_2 development: 1	none	1241[F]	9211[F]	11	11
NZW rabbit	embryofetal development / 0.01, 0.025, 0.05	Maternal: 0.05	none	22[G]	334[G]	0.4	0.4
		Fetal: < 0.01	fetal abnormalities	< 10[E]	< 137[E]	< 0.2	< 0 2
cyno monkey	52 weeks / 0.05, 0 5, 5	local toxicity: < 0.05	injection site reaction (not reversed)	NA[B]	NA[B]	< 0.01[H]	< 0.01[H]
		systemic toxicity : <0 05	inflammatory cell infiltrate in kidneys (F) and stomach pylorus (M & F)	< 46	< 817	< 1.0	< 1 0

[A]Human safety margins calculated by dividing plasma liraglutide $AUC_{0\text{-}24h}$ in animals by the $AUC_{0\text{-}24h}$ in humans at steady state in healthy adults at the maximum recommended human dose (MRHD) of 1.8 mg/day liraglutide for the treatment of type 2 diabetes mellitus ($AUC_{0\text{-}24h}$ 809 nM*h) or in obese subjects at the MRHD of 3 0 mg/day for wieght management ($AUC_{0\text{-}24h}$ 854 nM*h).

[B]Not applicable.

[C]Exposure multiple based on comparison of liraglutide concentration in the 1 mg/kg dose formulation in the mouse carcinogenicity study (0.2 mg/mL) and the liraglutide concentration in the clinical formulation (6 mg/mL).

[D]Exposure estimated from day 28 TK parameters (males and females combined) from a 4-week repeat dose toxicity study in rats.

[E]Maternal plasma exposure on the last TK sample day.

[F]Estimated ancestral F_0 maternal plasma exposures. F_1 generation exposed *in utero* and from milk during nursing period, F_2 generation never exposed.

[G]Estimated using gestation day 6 and 16 exposures from mated female NZW rabbits administered doses of 0.02 and 0.1 mg/kg.

[H]Exposure multiple based on comparison of liraglutide concentration in the 0 05 mg/kg dose formulation in the mouse carcinogenicity study (0.02 mg/mL) and the liraglutide concentration in the clinical formulation (6 mg/mL).

Liraglutide Safety Concerns

Safety Concerns Based on Nonclinical Findings

The following safety concerns were identified in nonclinical studies.

Carcinogenicity

Both the approved label for Victoza and the proposed label for Saxenda state liraglutide causes dose-dependent and treatment duration-dependent thyroid C-cell tumors (adenomas and/or carcinomas) at clinically relevant exposures in both genders of rats and mice. Malignant thyroid C-cell carcinomas were detected in rats and mice. It is unknown whether liraglutide will cause thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans, as the human relevance of liraglutide-induced rodent thyroid C-cell tumors could not be determined by clinical or nonclinical studies. Liraglutide is contraindicated in patients with a personal or family history of medullary thyroid carcinoma (MTC) or in patients with Multiple Endocrine Neoplasia syndrome type 2 (MEN 2).

In a 104-week carcinogenicity study in male and female CD-1 mice using doses of 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide administered by bolus subcutaneous injection, a dose-related increase in benign thyroid C-cell adenomas was seen in the 1.0 and the 3.0 mg/kg/day groups with incidences of 13% and 19% in males and 6% and 20% in females, respectively. C-cell adenomas did not occur in control groups or 0.03 and 0.2 mg/kg/day groups. Treatment-related malignant C-cell carcinomas occurred in 3% of females in the 3.0 mg/kg/day group. Thyroid C-cell tumors are rare findings during carcinogenicity testing in mice.

In a 104-week carcinogenicity study in male and female Sprague Dawley rats using doses of 0.075, 0.25 and 0.75 mg/kg/day liraglutide administered by bolus subcutaneous injection, a treatment-related increase in benign thyroid C-cell adenomas was seen in males in 0.25 and 0.75 mg/kg/day liraglutide groups with incidences of 12%, 16%, 42%, and 46% and in all female liraglutide-treated groups with incidences of 10%, 27%, 33%, and 56% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. A treatment-related increase in malignant thyroid C-cell carcinomas was observed in all male liraglutide-treated groups with incidences of 2%, 8%, 6%, and 14% and in females at 0.25 and 0.75 mg/kg/day with incidences of 0%, 0%, 4%, and 6% in 0 (control), 0.075, 0.25, and 0.75 mg/kg/day groups, respectively. Thyroid C-cell carcinomas are rare findings during carcinogenicity testing in rats.

Approval of Victoza included 2 post-marketing requirements (PMRs) further evaluating the effects of liraglutide on proliferative C-cell lesions in mice; one study evaluating liraglutide exposure requirements for eliciting C-cell tumors (PMR 1583-3) and a second mechanistic study evaluating signaling pathways mediating liraglutide's C-cell proliferative effects (PMR 1583-5). Study 210145 fulfilling Victoza PMR 1583-3 was a 104-week study in CD-1 mice subcutaneously administered liraglutide for 26 weeks followed by a 78 week recovery period to determine if transient exposure to liraglutide increases the lifetime risk of developing proliferative C-cell lesions. Mice treated with 3.0 mg/kg/day liraglutide for 25% of their total life span (26 weeks) were at an increased risk of developing proliferative thyroid C-cell lesions (preneoplastic focal C-cell hyperplasia in males and benign C-cell adenoma in females) for up to 78 weeks after treatment was stopped. Due to the low incidence of proliferative C-cell lesions in thyroid in male and female high dose recovery group mice and in concurrent control group male mice, a clear relationship to liraglutide treatment was not established for proliferative C-cell lesions in high dose recovery groups. Whether or not transient exposure to liraglutide increases the lifetime risk of proliferative C-cell lesions in mice could not be adequately addressed by study 210145 because of the uncertainty that proliferative C-cell lesions in high dose recovery groups were related to liraglutide treatment. Study 209306 fulfilling Victoza PMR 1583-5 was a

13-week repeat dose study of liraglutide in wild-type CD-1 mice and GLP-1R deficient (GLP1rKO) mice showing liraglutide induced thyroid C-cell hyperplasia and activation of ribosomal protein S6 in normal and hyperplastic C-cells were both GLP-1R-dependent in mice. Liraglutide did not activate the rearranged during transfection proto-oncogene (RET), a receptor tyrosine kinase that is constitutively activated by RET gene mutations in the majority of sporadic and familial MTCs in humans.

In clinical studies of liraglutide for the treatment of T2DM, there was no evidence that liraglutide caused MTC in humans. As part of the safety evaluation for Saxenda NDA 206321, a 13 August 2014 pharmacovigilance review of post-marketing safety information for Victoza authored by Drs. Debra Ryan and Carolyn Tabak in CDER's Division of Pharmacovigilance I identified 9 cases MTC in the FDA Adverse Event Reporting System (FAERS) database. Human MTC is rare. Based on clinical evidence confirming the diagnosis of MTC and a causality assessment performed by the Division of Oncology Products 2, 6 of these case reports were consistent with sporadic MTC and possibly related to liraglutide treatment. (b) (5)

According to the Victoza label, in the 104-week carcinogenicity study of 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide administered by bolus subcutaneous injection to male and female CD-1 mice, a treatment-related increase in fibrosarcomas occurred on the dorsal skin and subcutis, the body surface used for drug injection, in males in the 3 mg/kg/day group. Fibrosarcomas at or near the injection site were attributed to the high local concentration of drug, but the liraglutide concentration in the clinical formulation (6 mg/mL) is 10-times higher than the concentration in the formulation used to administer 3 mg/kg/day liraglutide to mice in the carcinogenicity study (0.6 mg/mL).

According to the Victoza label, including clinical studies evaluating the use of liraglutide for the treatment of T2DM, there incidence of papillary thyroid carcinoma was 3-times greater in patients treated with liraglutide compared to comparator-treated patients. In the FDA briefing document for the 11 September 2014 meeting of the Endocrinologic and Metabolic Drug Advisory Committee reviewing safety and efficacy of liraglutide for weight management, a numerical imbalance not favoring liraglutide for breast cancer was noted by Dr. Julie Golden, the clinical reviewer.

Reproductive and Developmental Toxicity

According to the Victoza label and the proposed Saxenda label, liraglutide was teratogenic in rats and rabbits at clinically relevant exposures.

In female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide beginning 2 weeks before mating through gestation day 17, the number of early embryonic deaths in the 1 mg/kg/day group increased slightly. Fetal abnormalities and variations in kidneys and blood vessels, irregular ossification of the skull, and a more complete state of ossification occurred at all doses. Mottled liver and minimally kinked ribs occurred at the highest dose. The incidence of fetal malformations in liraglutide-treated groups exceeding concurrent and historical controls were misshapen oropharynx and/or narrowed opening into larynx at 0.1 mg/kg/day and umbilical hernia at 0.1 and 0.25 mg/kg/day.

In pregnant rabbits given subcutaneous doses of 0.01, 0.025 and 0.05 mg/kg/day liraglutide from gestation day 6 through day 18, liraglutide decreased fetal weight and dose-dependently increased the incidence of total major fetal abnormalities at all doses. The incidence of malformations exceeded concurrent and historical controls at 0.01 mg/kg/day (kidneys, scapula), ≥ 0.01 mg/kg/day (eyes, forelimb), 0.025 mg/kg/day (brain, tail and sacral vertebrae, major blood vessels and heart, umbilicus), ≥ 0.025 mg/kg/day (sternum) and at 0.05 mg/kg/day (parietal bones, major blood vessels). Irregular ossification and/or skeletal abnormalities occurred in the skull and jaw, vertebrae and ribs, sternum, pelvis, tail, and scapula; and dose-dependent minor skeletal variations were observed. Visceral abnormalities

occurred in blood vessels, lung, liver, and esophagus. Bilobed or bifurcated gallbladder was seen in all treatment groups, but not in the control group.

In pregnant female rats given subcutaneous doses of 0.1, 0.25 and 1.0 mg/kg/day liraglutide from gestation day 6 through weaning or termination of nursing on lactation day 24, a slight delay in parturition was observed in the majority of treated rats. Group mean body weight of neonatal rats from liraglutide-treated dams was lower than neonatal rats from control group dams. Bloody scabs and agitated behavior occurred in male rats descended from dams treated with 1 mg/kg/day liraglutide. Group mean body weight from birth to postpartum day 14 trended lower in F_2 generation rats descended from liraglutide-treated rats compared to F_2 generation rats descended from controls, but differences did not reach statistical significance for any group.

A Written Request for Pediatric Studies issued under NDA 22341 for Victoza requires Novo Nordisk to (b) (4)

Safety Concerns Based on Clinical Findings

The following safety concerns in the approved label for Victoza or the proposed label for Saxenda are based on findings from clinical studies.

Pancreatitis

According to the label for Victoza, in clinical studies, the incidence of pancreatitis was 5.4-times higher in Victoza-treated patients than in comparator-treated patients (glimepiride) and reports of acute pancreatitis, including fatal and non-fatal hemorrhagic or necrotizing pancreatitis in patients treated with liraglutide were reported post-marketing. The proposed label for Saxenda states in (b) (4) Review of repeat dose toxicity studies of liraglutide submitted to Victoza NDA 22341, including chronic toxicity studies in rats and monkeys and carcinogenicity studies in mice and rats, did not show any substantive evidence of pancreatitis or pancreatic cancer.

Based on a higher incidence of pancreatitis in Victoza-treated patients and a published study suggesting incretin-acting drugs may cause pancreatitis in a human islet amyloid polypeptide transgenic mouse model of T2DM (Matveyenko et al. Diabetes. 2009 Jul;58(7):1604-15), approval of Victoza included a post-marketing requirement to determine the effects of liraglutide on the exocrine pancreas in a rodent model of insulin-resistant T2DM (PMR 1583-4). Study LoSi100801 titled “A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes” fulfilling PMR 1583-4 was reviewed under Victoza NDA 22341. In this study, up to 1.0 mg/kg/day liraglutide for 3 months had no adverse effects on the exocrine pancreas of male or female hyperglycemic Zucker Diabetic Fatty (ZDF) fa/fa rats.

In Saxenda NDA 206321, the applicant reported microscopic evaluation of pancreas from cynomolgus monkeys treated with 0, 0.25, or 5 mg/kg/day liraglutide for 87 weeks from a thyroid safety study titled “Effects on calcium homeostasis related parameters after up to 87 weeks daily subcutaneous administration in male and female cynomolgus monkeys – combined

evaluation of in life phase including thyroid histopathological evaluation". This study report was previously submitted in the 2008 annual report for IND 61,040 investigating the use of liraglutide in patients with T2DM during the review of Victoza NDA 22341. The applicant considered minimal focal inflammation in the exocrine pancreas occurring in 3/5 male cynomolgus monkeys treated with 5 mg/kg/day liraglutide for 87 weeks background findings because findings were not consistent (perivascular inflammation in 1 male, parenchymal inflammation and minimal focal lobular atrophy in a second male, and ductal inflammation in a third), there were no findings in females in the same dose group, and there were no findings in males or females treated with 0.25 mg/kg/day liraglutide. A second pancreas safety study report finalized in March 2009 titled "Evaluation of pancreas from high dose animals from toxicity studies in cynomolgus macaques against diagnostic criteria for PanIN" was submitted to Saxenda NDA 206321. Microscopic evaluation of pancreas from high dose group cynomolgus monkeys in 4-, 13-, 52-, and 87-week repeat dose studies of liraglutide against criteria for pancreatic intraepithelial neoplasia (PanIN) in small caliber ducts showed there were no PanIN lesions present in pancreas from any monkey treated with 5 mg/kg/day liraglutide for up to 87 weeks. Novo Nordisk published their findings from macroscopic and microscopic examination of pancreas from cynomolgus monkeys treated with liraglutide, a lipidated GLP-1 analog subcutaneously injected once a day for 4, 13, 52, or 87 weeks or treated with semaglutide, a lipidated GLP-1 analog subcutaneously injected once a week for 13 or 52 weeks. There were no adverse effects of liraglutide or semaglutide in pancreas of cynomolgus monkeys (Gotfredsen et al, Diabetes 2014;63:2486–2497).

A recently published study showed liraglutide increased the number of pancreatic β cells and increased proliferation of small duct epithelial cells in male WBN/Kob(fa) (fa/fa) rats (7 weeks old) subcutaneously administered 0 (saline), 0.038, 0.075, or 0.15 mg/kg/day liraglutide once a day for 4 weeks, (Nagakubo et al, Comp Med. 2014 Apr;64(2):121-7). Liraglutide upregulated GLP-1Rs, inhibited growth, and promoted apoptosis in human pancreatic cancer cell lines *in vitro* (MIA PaCa-2 and PANC-1) and inhibited growth of human pancreatic tumors produced by injecting MIA PaCa-2 cells into nude mice (Zhoa et al, Am J Physiol Endocrinol Metab 306: E1431–E1441, 2014).

Consistent with the absence of evidence of pancreas injury in repeat dose toxicity studies of liraglutide in mice, rats, and monkeys, an animal model of GLP-1R agonist-induced pancreatitis has not been established. A recent published perspective from the FDA and the European Medicines Agency (EMA) concludes nonclinical data, clinical data, and post-marketing data for liraglutide and other incretin-based drugs are not sufficient to establish a causal relationship between the use of incretin-based drugs and pancreatitis or pancreatic cancer (Egan et al. N Engl J Med. 2014 Feb 27;370(9):794-7).

Renal Impairment

According to the label for Victoza, there were post-marketing reports of acute renal failure and worsening chronic renal failure in patients treated with liraglutide. Impaired renal function in liraglutide treated patients usually occurred in patients that experienced nausea, vomiting, diarrhea, or dehydration. The proposed label for Saxenda includes a warning about drug-related renal impairment based on clinical studies of Saxenda and Victoza post-marketing reports. (b) (4)

No new nonclinical studies evaluating renal toxicity of liraglutide were reported after approval of Victoza. In study LoSi100801 titled "A 3 month study of the effects of liraglutide on the exocrine pancreas in a rodent model of insulin resistant type 2 diabetes" fulfilling PMR 1583-4 reviewed under Victoza NDA 22341, male or female hyperglycemic Zucker Diabetic Fatty (ZDF) fa/fa rats were subcutaneously administered 0 (vehicle), 0.4, or 1.0 mg/kg/day liraglutide administered once a day or 1.0 mg/kg/day liraglutide administered twice a day (0.5

mg/kg/injection) for 13 weeks. Three of 8 diabetic male rats in a vehicle control group mistakenly administered a single subcutaneous dose of 0.5 mg/kg liraglutide on day 17 were found dead within 5 days of dosing with clinical signs of dehydration and apathy prior to death. Although clinical signs were consistent with renal failure as a possible cause of death, subcutaneous saline administered to treat dehydration after misdosing with liraglutide did not prevent mortality, serum chemistry parameters related to kidney function were not evaluated, and a cause of death was not identified by limited necropsies of 2/3 decedents.

Hypersensitivity

According to the Victoza label, serious hypersensitivity reactions, including anaphylaxis and angioedema, have occurred in patients treated with liraglutide and angioedema was reported in patients treated with other GLP-1R agonists. Liraglutide is contraindicated in patients with a history of serious hypersensitivity to liraglutide or any product components. No new nonclinical studies evaluating liraglutide hypersensitivity were performed after approval of Victoza NDA 22341 in January 2010.

Hypoglycemia

According to the Victoza label, combined treatment with liraglutide and an insulin secretagogue or insulin may increase the risk of hypoglycemia. No new nonclinical studies evaluating hypoglycemic effects of liraglutide coadministered with insulin or insulin secretagogues were submitted in Saxenda NDA 206321.

Injection Site Reactions

According to the Victoza label, injection site reactions occurred in approximately 2% of liraglutide-treated patients, and <0.2% of liraglutide-treated patients discontinued treatment due to injection site reactions. According to the proposed Saxenda label, injection site reactions occurred in (b) (4)% of Saxenda-treated patients compared to (b) (4)% of placebo-treated patients, but discontinuation due to injection site reactions was (b) (4)%.

According to the Victoza label, in the 104-week carcinogenicity study of 0.03, 0.2, 1.0, and 3.0 mg/kg/day liraglutide administered by bolus subcutaneous injection to male and female CD-1 mice, a treatment-related increase in fibrosarcomas occurred on the dorsal skin and subcutis, the body surface used for drug injection, in males in the 3 mg/kg/day group. Fibrosarcomas at or near the injection site were attributed to the high local concentration of drug, but the liraglutide concentration in the clinical formulation (6 mg/mL) is 10-times higher than the concentration in the formulation used to administer 3 mg/kg/day liraglutide to mice in the carcinogenicity study (0.6 mg/mL).

Two studies evaluating local toxicity of liraglutide were submitted to Saxenda NDA 206321. The first study investigated local toxicity of the “to-be-marketed” formula in pigs and the second study in rabbits evaluated local toxicity due to errant dosing. In LYD pigs subcutaneously injected on the dorsal surface with saline (0.9% sodium chloride), vehicle (1.42 mg/mL disodium phosphate dehydrate, 5.5 mg/mL phenol, 14.0 mg/mL phenol, (b) (4) pH to 8.15), or the to-be-marketed formulation of liraglutide (6.0 mg/mL liraglutide in vehicle), injection site reactions characterized by inflammation, hemorrhage, and fibroblast activation persisted for up to 5 days after dosing. In a single dose study in rabbits evaluating effects of misdosing with 0.06 mL vehicle or liraglutide (6 mg/mL, clinical formulation) by intramuscular (thigh), intravenous (ear), or intra-arterial (ear) injection, injection site reactions occurred after intramuscular injection (microscopic focal accumulation of mononuclear cells and skeletal muscle fiber degeneration / regeneration in both, but greater at sites injected with liraglutide), intravenous injection (necrosis of the vessel wall, perivascular necrosis, hemorrhage, and accumulation of inflammatory cells in both vehicle and liraglutide injection sites that resolved within 5 days of dosing), and intra-arterial injection (swelling, erythema, and

hemorrhage occurred in both vehicle and liraglutide injection sites, the injection site reactions were more severe at liraglutide injection sites and did not resolve within 5 days after dosing with macroscopic hemorrhage and correlative microscopic perivascular hemorrhage, edema, and inflammation persisting).

Immunogenicity

According to the Victoza label, in clinical studies of liraglutide, anti-drug antibodies (ADAs) were detected in 8.6% of liraglutide-treated patients, ADAs cross-reacted with GLP-1 in 4.8% - 6.9% of liraglutide-treated patients, and ADAs were neutralizing in an *in vitro* assay in 1.0% - 2.3% of liraglutide-treated patients. Patients that developed ADAs were not more likely to develop adverse events related to immunogenicity, such as urticarial or angioedema. According to the proposed Saxenda label, (b) (4)% of Saxenda-treated patients developed ADAs.

Heart Rate Increase

The Victoza label states liraglutide increased heart rate 2 to 3 beats per minute (bpm) compared to baseline, without adversely affecting blood pressure, and the long-term clinical effects of the increase in pulse rate were not established. According to the proposed Saxenda label, mean heart rate was increased 2 to 3 beats per minute (bpm) compared to baseline in liraglutide-treated patients, the clinical significance of increased heart rate is not known, and liraglutide should be discontinued in patients experiencing a sustained increased heart rate while taking liraglutide.

No new nonclinical studies evaluating cardiovascular effects of liraglutide were reported after the approval of Victoza. Nonclinical studies previously reviewed under Victoza NDA 22341 showed liraglutide increased heart rate *ex vivo* in isolated rabbit heart preparation (1.43 µM) and *in vivo* in a single dose cardiovascular safety study in rats ($\geq$0.2 mg/kg) and in a pig model of myocardial infarction (0.01 mg/kg for 3 days), but liraglutide had no effect on heart rate in repeat dose toxicity studies in cynomolgus monkeys treated for up to 52 weeks. A published study evaluating GLP-1R tissue distribution in monkeys and humans by immunohistochemical staining using a receptor specific monoclonal antibody and *in situ* radioligand binding showed the GLP-1R was expressed in myocytes of the sino-atrial node and smooth muscle cells in arteries and arterioles in kidney (Pyke et al. Endocrinology. 2014 Apr;155(4):1280-90). Although GLP-1Rs were present in sino-atrial node myocytes in heart from both humans and monkeys, in the absence of an effect of liraglutide on heart rate in monkeys, it is unclear if GLP-1Rs in the sino-atrial node mediate the effects of liraglutide on heart rate in humans.

Cholelithiasis and Cholecystitis

Increased incidences of cholelithiasis and cholecystitis were noted in liraglutide-treated subjects in clinical studies of >1.8 mg/day liraglutide for weight management, but not in clinical studies of $\leq$1.8 mg/day liraglutide for the treatment of T2DM. The proposed label for Saxenda states that in clinical studies of liraglutide for weight management, the reported incidence of cholelithiasis and cholecystitis was 3-times higher in liraglutide-treated subjects compared to placebo-treated subjects (1.5% Saxenda-treated patients compared to 0.5% placebo-treated patients for cholelithiasis and 0.6% Saxenda-treated patients compared to 0.2% of placebo-treated patients for cholelithiasis).

In repeat dose toxicity studies, up to 5 mg/kg/day liraglutide had no effect on gall bladder of mice treated for up to 13 weeks or cynomolgus monkeys treated for up to 52 weeks. In the 104 week carcinogenicity study of liraglutide in mice, liraglutide increased the incidence of macroscopic pathology findings in gall bladder including abnormal contents, dilation / distension, and enlarged at all doses compared to control, but in general, these macroscopic findings lacked a relation to liraglutide dose or correlative microscopic pathology (summary table below).

Incidence of Macroscopic Finding in Gall Bladder in 104-Week Mouse Carcinogenicity Study

Sex		Male					Female				
Liraglutide Dose (mg/kg/day)		0	0.03	0.2	1	3	0	0.03	0.2	1	3
Gall Bladder Finding	N[1]	96	84	84	84	96	96	84	84	84	96
abnormal contents	N	0	4	2	7	7	0	1	5	4	7
	%	0.0	4.8	2.4	8.3	7.3	0.0	1.2	6.0	4.8	7.3
dilated / distended	N	1	2	3	7	6	4	8	7	15	12
	%	1.0	2.4	3.6	8.3	6.3	4.2	9.5	8.3	17.9	12.5
enlarged	N	1	6	9	9	3	1	8	10	7	11
	%	1.0	7.1	10.7	10.7	3.1	1.0	9.5	11.9	8.3	11.5

[1]All groups terminated at the end of the study combined including main study group, 78 week interim sacrifice groups rescheduled for termination at end of study due to reduced survival of main study groups, and week 104 satellite toxicokinetic groups

Table 3-1 (below) shows the incidence of cholecystitis exceeded the incidence in concurrent and historical control groups at 3.0 mg/kg/day liraglutide in male mice and at 0.03 and 1.0 mg/kg/day in females with a statistically significant higher incidence compared to control at 0.03 mg/kg/day liraglutide in females.

Table 3–1 Incidences of cholecystitis and cholelithiasis in 104-week mouse carcinogenicity study

Sex	Group (mg/kg/day)	Dose Group (mg/kg/day) 0	0.03	0.2	1.0	3.0	Historical control range
	Exposure multiples animal:human	-	0.2	1.7	14	40	
Males	Number examined	72	59	58	65	68	
	Cholelithiasis, n (%)	0	0	0	0	0	0 – 1.7 %
	Cholecystitis, n (%)	0	2 (3.4)	2 (3.4)	1 (1.5)	3 (4.4)	0 – 3.4 %
Females	Number examined	71	60	61	58	71	
	Cholelithiasis, n (%)	0	0	0	1.7	1.4	0 – 6.8 %
	Cholecystitis, n (%)	0	4 (6.6*)	1 (1.6)	2 (3.4)	0 (0)	0 – 3.3 %

*p<0.05 vs control. Historical control range is the incidences of findings recorded at test laboratory where study was conducted.

[NDA 206321 EMDAC Briefing Document from Novo Nordisk, P36]

In a repeat dose study evaluating the effects of 1 mg/kg exenatide subcutaneously injected 3 times a day for 12 weeks on plasma calcitonin in CD-1 mice (study 205025), exenatide caused macroscopic distended gall bladder in females. Continuous subcutaneous infusion of exenatide in CD-1 mice caused a low incidence of macroscopic gall bladder distension in males (at 0.25 mg/kg/day in week 12 and at 0.25 and 1.0 mg/kg/day in weeks 12 and 16) and females (at 0.25 and 1.0 mg/kg/day in weeks 12 and 16).

A published study of the effects of exenatide on cholecystokinin-induced gall bladder emptying in humans showed exenatide reduced CCK-induced gall bladder emptying in fasting healthy subjects (Keller et al, Regul Pept. 2012 Nov 10;179(1-3):77-83).

Central Nervous System Effects

The proposed label for Saxenda states that in clinical studies of liraglutide for weight management, asthenia, fatigue, malaise, dysgeusia, and dizziness were (b) (4) co-reported with nausea, vomiting, and diarrhea (b) (4) the first 12 weeks of treatment.

During the development of liraglutide for weight management under IND 73,206, Novo Nordisk requested a consult from CDER's Controlled Substances Staff to determine if abuse potential studies would be required and in a September 2012 Advice Letter, the Agency agreed abuse potential studies would not be required because there was no evidence of potential abuse signals in nonclinical toxicity studies, adverse event reports from clinical studies of liraglutide monotherapy, and quarterly adverse effects reports for Victoza from April 2010 to January 2012 and no evidence of abuse potential in articles of basic or clinical studies of liraglutide or review articles appearing in PubMed between 1994 and 2012. The GLP-1R agonist exenatide attenuated amphetamine- or cocaine-induced locomotor stimulation, accumbal dopamine release, and conditioned place preference in mice indicating it attenuates the rewarding properties of psychostimulant drugs (Egecioglu et al, PLoS One. 2013 Jul 16;8(7):e69010)

Studies evaluating distribution of subcutaneously administered liraglutide to the central nervous system (CNS) in rats reviewed under Victoza NDA 22341 showed poor penetration of liraglutide into the CNS of albino or pigmented rats. In normal animals, liraglutide transiently reduced food consumption at the beginning of treatment in mice, rats, and monkeys, but a sustained reduction of body weight gain with continued treatment only consistently occurred in male rats and male monkeys. Liraglutide decreased food consumption in animal models of obesity including candy fed diet-induced obese Sprague Dawley rats, 8 month old diet-induced obese Sprague Dawley rats, Wistar rats with monosodium glutamate-induced deficits in arcuate nucleus GLP-1R signaling, and obese diabetic *ob/ob* mice, but not in Zucker diabetic fatty rats. A published study showed liraglutide reduced food consumption of WBN/Kob-Lepr(fa (fa/fa) rats, a model of type 2 diabetes with obesity and chronic pancreatitis, but without decreasing body weight gain (Nagakubo et al, Comp Med. 2014 Apr;64(2):121-7).

In NDA 206321, the applicant submitted additional reports of studies evaluating liraglutide access to the brain, activation of neurons in specific brain regions, and parts of the nervous system mediating its anorexic effects. These studies showed subcutaneously administered liraglutide accesses circumventricular brain areas in mice and rats with liraglutide-related material detected in the choroid plexus and zona externa of the median eminence (not dependent on GLP-1R expression) and in circumventricular regions and the hypothalamus (GLP-1R-dependent). In rats, liraglutide-related material colocalized with cocaine and amphetamine related transcript (CART) neurons in the hypothalamic arcuate nucleus. In rats, liraglutide increased activation of neurons in the area postrema and lateral parabrachial nucleus of fasted and fed rats and increased activation of dopaminergic and/or adrenergic neurons in the area postrema of fed rats, but not fasted rats. Liraglutide activated non-GLP-1 neurons and reduced activation of GLP-1 neurons in the nucleus of the solitary tract and central amygdala and decreased the number and proportion of activated CART neurons in the arcuate nucleus of fed rats, but not fasted rats. Specific areas of the nervous system mediating liraglutide-induced decreased food consumption were not identified by the absence of a response to liraglutide to reduce food consumption or body weight gain in rats with lesions of afferent vagus nerves, brain stem area postrema, or hypothalamic paraventricular nucleus. A published study using mice with reduced GLP-1R in the central nervous system or peripheral nervous system indicate the central nervous system mediates the anorexic effects of liraglutide because liraglutide reduced food consumption in peripheral nerve GLP-1R knockdown mice, but not in CNS GLP-1R knockdown mice (Sisley et al, J Clin Invest. 2014 Jun 2;124(6):2456-63) and a second study

indicates the lateral parabrachial nucleus mediates anorexic effects of GLP-1R agonists (Richard et al. Endocrinology. 2014 Aug 13:en20141248 [Epub ahead of print]).

12 Appendix/Attachments

Appendix 1: Assessment of [redacted] and [redacted] genetic toxicity from Novo Nordisk

Novo Nordisk submitted the following information (SDN 29 received 15 July 2015) in response to a 1 July 2014 information request.

1.2 Question 2

Please provide a tabulated summary of all genotoxicity data for [redacted] establishing that it is not genotoxic.

1.2.1 Response to Question 2

A summary table for all Novo Nordisk sponsored genotoxicity analyses and studies is provided in Table 5.

No data was found in the publicly available scientific literature on the genotoxic potential of [redacted] and [redacted] Therefore, Novo Nordisk performed *in silico* and *in vivo* analyses to support the safety evaluation.

In a combined *in silico* analysis using DEREK Nexus and Leadscope, no structural alerts for mutagenic potential were identified for [redacted] Therefore, and in accordance with the ICH M7 guidance (Section 6 Table 1) [redacted] should be treated as a non-mutagenic substance. This is consistent with published test results for other [redacted] [1].

The combined *in silico* analysis showed a positive alert for *in vitro* clastogenicity for both [redacted] and [redacted] Therefore, two *in vivo* bone marrow micronucleous tests were carried out in mice. No clastogenic potential was observed in any of these studies in doses up to 400 mg/kg/day.

Based on the above listed information, [redacted] are considered not genotoxic.

1. [redacted]

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Appendix 2: Assessment of [redacted] (b) (4) and [redacted] (b) (4) genetic toxicity from the Chemical Informatics Group at FDA's Center for Drug Evaluation and Research

To: Tony Parola
cc: Karen Davis-Bruno
From: CDER/OTS/OCP/DARS: The Chemical Informatics Group
Re: NDA 206321
Date: July 10, 2014

Two leachables have been evaluated by CDER/OTS/OCP/DARS for genetic toxicity using (quantitative) structure-activity relationship [(Q)SAR] models. Four software programs were used: *Derek Nexus* 4.0.5 (*DX*), *Leadscope Model Applier* 1.8.3-1 (*LMA*), and *MC4PC* 2.4.1.4 (*MC*) or *CASE Ultra* 1.4.6.6 (*CU*). To maximize sensitivity and negative predictivity, a positive prediction from any one software program was used to justify a positive overall call.

---

1. [redacted] (b) (4)

Salmonella Mutagenicity[1]

[redacted] (b) (4)	Software	*Salmonella* Mutagenicity
	Derek Nexus	-
	Leadscope Model Applier	-
	CASE Ultra	-
	Overall Software Prediction	-
	Overall Expert Prediction	**-**

[redacted] (b) (4) is predicted to be negative for *Salmonella* mutagenicity.

Genetic Toxicity for Predicting the ICH S2 Battery[1]

Software	*Salmonella* Mutagenicity	*E. coli* Mutagenicity	Mouse Lymphoma	*In Vitro* Chromosome Aberrations	*In Vivo* Micronucleus
Derek Nexus	-	-	NSA	+	NSA
Leadscope Model Applier	-	-	NC	-	NC
CASE Ultra/MC4PC	-	-	-	-	-
Overall Software Prediction	-	-	NC	+	NC
Overall Expert Prediction	**-**	**-**	NC	**+**	NC

[redacted] (b) (4) is predicted to be negative for bacterial mutagenicity. In contrast, [redacted] (b) (4) is predicted to be positive for *in vitro* chromosome aberrations based solely on a positive prediction from *Derek Nexus*. (b) (4) [redacted]

[1] + = positive; – = negative; Eqv = equivocal; NSA = no structural alerts are identified by DX (Derek Nexus cannot differentiate between a negative call and the inability to make a call because of no coverage); NC = test chemical features are not adequately represented in the model training data set, leading to a no call.

2. (b) (4)

Salmonella Mutagenicity[1]

(b) (4)	Software	*Salmonella* Mutagenicity
	Derek Nexus	-
	Leadscope Model Applier	-
	CASE Ultra	-
	Overall Software Prediction	-
	Overall Expert Prediction	**-**

(b) (4) is predicted to be negative for *Salmonella* mutagenicity.

Genetic Toxicity for Predicting the ICH S2 Battery[1]

Software	*Salmonella* Mutagenicity	*E. coli* Mutagenicity	Mouse Lymphoma	*In Vitro* Chromosome Aberrations	*In Vivo* Micronucleus
Derek Nexus	-	-	NSA	+	NSA
Leadscope Model Applier	-	-	NC	-	NC
CASE Ultra/MC4PC	-	-	-	-	Eqv
Overall Software Prediction	-	-	NC	+	NC
Overall Expert Prediction	**-**	**-**	NC	**+**	NC

(b) (4) is predicted to be negative for bacterial mutagenicity. In contrast (b) (4) is predicted to be positive for *in vitro* chromosome aberrations by *Derek Nexus* (b) (4).

This report has been reviewed and approved by CDER/OTS/OCP/DARS.

Appendix 3: Consult from CDER's Pharmacology/Toxicology Coordinating Committee Genetic Toxicology Subcommittee regarding the validity and outcome of *in vivo* micronucleus assays of [redacted] and [redacted] in mice.

MEMORANDUM

Date: July 13, 2014

To: Anthony L. Parola, Ph.D.
Pharmacologist
Division of Metabolism and Endocrinology Products

Karen L. Davis-Bruno, Ph.D.
Supervisory Pharmacologist
Division of Metabolism and Endocrinology Products

Cc: Drs. Aisar Atrakchi (Division of Psychiatry Products), Abigail Jacobs (Office of New Drugs), and Tim McGovern (Office of New Drugs),

From: Mark W. Powley, Ph.D.
Co-Chair, Genetic Toxicology Subcommittee

Subject: Genotoxicity of [redacted]

Background

This consult addresses the *in vivo* micronucleus (MN) evaluation of [redacted] and [redacted] are impurities of liraglutide, a lipidated peptide analog of GLP-1 approved for the treatment of type 2 diabetes that is also being developed for weight management. The impurities are produced from [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Summary

MN formation was evaluated in male CD-1 mice administered subcutaneous injections of 100, 200, and 400 mg/kg/day of [redacted] or [redacted] for 2 days. Negative control groups included both untreated and vehicle treated animals (polyethylene glycol 400 for [redacted] 10% ethanol in polyethylene glycol 400 for [redacted] Positive controls received a single oral dose of 40 mg/kg cyclophosphamide. Clinical signs indicative of significant toxicity

(e.g., decreased activity) were observed at the high-dose suggesting a reasonable approximation of the MTD had been achieved. While the % polychromatic erythrocytes (PCE) were similar in treated and concurrent controls groups, individual values were below the historical control mean and the 95% confidence interval range. The discrepancy was described as the result of sub-optimal staining leading to difficulty in differentiating between PCE and normochromatic erythrocytes (NCE). In spite of these technical difficulties, the testing laboratory expressed confidence that an appropriate number of PCE were identified for MN analysis. Overall, there were no test article-related effects on MN formation at the doses tested (data summarized in the Appendix).

It is also noteworthy that 2 out of the 6 positive control animals in the (b) (4) study were excluded from analysis due to the impact of cell clumping on MN analysis. The testing laboratory relied on the remaining positive control data to confirm the expected response.

Question from the Division of Metabolism and Endocrinology Products

1. Does the subcommittee agree that the *in vivo* micronucleus assays of (b) (4) and (b) (4) are not valid?

   Subcommittee Response – No, the responding subcommittee members considered the studies to be valid and acceptable for regulatory decision making.

2. If the subcommittee believes the assays are valid, are (b) (4) and (b) (4) negative in the *in vivo* micronucleus assays?

   Subcommittee Response – Yes, the assays appear to be negative.

Summary of Genetic Toxicology Subcommittee Comments

- The responding subcommittee members shared the reviewer's concerns with validity; however, the concerns were not deemed sufficient to disregard study results. It was acknowledged that the studies may not have been considered acceptable for characterizing an API.
- While the %PCE for vehicle and untreated control animals fell below the historical control mean and 95% confidence interval range, individual values were within the observed historical control range. Overall, concurrent control values were useful for confirming a lack of test article-related bone marrow toxicity.
- Cell-clumping prevented accurate assessment of 2 positive control animals in the (b) (4) study. The 4 remaining animals were within the observed range as well as 95% confidence interval range for historical controls and were considered sufficient to confirm the expected positive control article response.
- The Sponsor's decision to conduct an *in vivo* assay instead of (Q)SAR or an empirical Ames assay was also of interest. (b) (4) closely related structural analogs) are primarily reported as Ames negative and it is questionable whether (Q)SAR would have predicted (b) (4) and (b) (4) to be mutagenic. Depending on the potential exposures to (b) (4) and (b) (4) (Q)SAR alone could have been enough to qualify the impurities.

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/s/

ANTHONY L PAROLA
09/13/2014

KAREN L DAVIS BRUNO
09/15/2014
concur

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY NDA REVIEW AND EVALUATION

Application number:	206,321
Supporting document/s (CDER stamp date):	00 / 12.20.13 / (b) (4)
Product:	Saxenda (6 mg/mL liraglutide (rDNA origin) for injection)
Indication:	(b) (4)
Applicant:	Novo Nordisk, PO Box 846, Plainsboro, NJ 08536
Review Division:	Metabolism and Endocrinology Products
Reviewer:	Anthony Parola, PhD
Supervisor/Team Leader:	Karen Davis-Bruno, PhD
Division Director:	Jean-Marc Guettier, MD
Project Manager:	Patricia Madara

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PHARMACOLOGY/TOXICOLOGY FILING CHECKLIST FOR NDA/BLA or Supplement

NDA Number: 206,321 **Applicant:** Novo Nordisk Inc. **Stamp Date:** 12/20/13

Drug Name: Saxenda™ (6 mg/mL liraglutide injection) **NDA Type:** 505(b)(1) cross referencing NDA 22-341 (Victoza®), IND 73,206 (liraglutide for weight management), and DMFs (b) (4)

On **initial** overview of the NDA/BLA application for filing:

	Content Parameter	Yes	No	Comment
1	Is the pharmacology/toxicology section organized in accord with current regulations and guidelines for format and content in a manner to allow substantive review to begin?	X		NDA 206,321 references required nonclinical studies previously submitted to module 4 of NDA 22,341 (Victoza®). However, specific files containing the referenced information are not identified by file name, reference number, and page number in the Agency's record where the information can be found.
2	Is the pharmacology/toxicology section indexed and paginated in a manner allowing substantive review to begin?	X		
3	Is the pharmacology/toxicology section legible so that substantive review can begin?	X		
4	Are all required (*) and requested IND studies (in accord with 505 b1 and b2 including referenced literature) completed and submitted (carcinogenicity, mutagenicity, teratogenicity, effects on fertility, juvenile studies, acute and repeat dose adult animal studies, animal ADME studies, safety pharmacology, etc)?	X		No nonclinical studies were requested under IND 73,206. Required nonclinical studies submitted to NDA 22,341 supporting the approved marking application for Victoza® are referenced by NDA 206,321 for Saxenda™.
5	If the formulation to be marketed is different from the formulation used in the toxicology studies, have studies by the appropriate route been conducted with appropriate formulations? (For other than the oral route, some studies may be by routes different from the clinical route intentionally and by desire of the FDA).	X		The to be marketed formulation of 6 mg/mL liraglutide in Saxenda™ is identical to the marketed formulation in Victoza®.
6	Does the route of administration used in the animal studies appear to be the same as the intended human exposure route? If not, has the applicant submitted a rationale to justify the alternative route?	X		

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	Content Parameter	Yes	No	Comment
7	Has the applicant submitted a statement(s) that all of the pivotal pharm/tox studies have been performed in accordance with the GLP regulations (21 CFR 58) or an explanation for any significant deviations?	-	-	Not applicable. Pivotal nonclinical studies were reviewed under NDA 22,341 for Victoza®.
8	Has the applicant submitted all special studies/data requested by the Division during pre-submission discussions?	-	-	Not applicable. No special nonclinical studies or data were requested.
9	Are the proposed labeling sections relative to pharmacology/toxicology appropriate (including human dose multiples expressed in either mg/m2 or comparative serum/plasma levels) and in accordance with 201.57?	X		
10	Have any impurity – etc. issues been addressed? (New toxicity studies may not be needed.)	X		(b) (4) [redacted]. The PDS290 pen injector is a new device that was not used in phase 3 clinical studies of liraglutide. The sponsor evaluated leachables from the container closure system.
11	Has the applicant addressed any abuse potential issues in the submission?	X		In September 2013, the Controlled Substances Staff recommended nonclinical abuse potential studies for liraglutide are not warranted.
12	If this NDA/BLA is to support a Rx to OTC switch, have all relevant studies been submitted?	-	-	Not applicable.

IS THE PHARMACOLOGY/TOXICOLOGY SECTION OF THE APPLICATION FILEABLE? YES

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter. None

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This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

ANTHONY L PAROLA
02/05/2014

KAREN L DAVIS BRUNO
02/05/2014